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ABSTRACT
Resistance to Imatinib mesylate is emerging as a real clinical problem in the management of chronic myelogenous leukemia (CML). In this project, we are exploring the hypothesis that epigenetic silencing associated with promoter DNA methylation mediates resistance in selected cases, and that reversal of silencing by decitabine-induced hypomethylation can be of therapeutic benefit in CML. In progress to date, we have identified samples from patients with CML prior to Imatinib therapy, as well as from patients with established resistance to Imatinib. Bisulfite based analysis identified methylation of p15 and CDH13 in subsets of patients but ruled these genes out as major causes of resistance. In parallel, clinical trials of decitabine have shown activity as single agent and when combined with Imatinib in CML resistant to Imatinib. Analysis of samples from patients on trial showed hypomethylation after therapy. Hypomethylation dynamics suggest that decitabine leads to CML cell death 5-10 days after treatment and suggest that resistance to decitabine is not pharmacologic. These studies are ongoing to clarify the role of methylation in the pathogenesis and therapy of Imatinib resistant CML.

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Introduction

Resistance to Imatinib mesylate is emerging as a real clinical problem in the management of chronic myelogenous leukemia (CML)[1]. In this project, we are exploring the hypothesis that epigenetic silencing associated with promoter DNA methylation mediates resistance in selected cases, and that reversal of silencing by decitabine-induced hypomethylation can be of therapeutic benefit in CML. The original plan called for (1) identifying samples from patients with CML treated with imatinib and examining DNA methylation in relation to resistance and (2) examining, in the setting of clinical trials, the effects of the hypomethylating drug decitabine on DNA methylation and gene expression in imatinib resistant CML, and correlations of these molecular marks with treatment responses.

Body

Progress on task 1:

The CML database at MD Anderson was queried, and data on over 500 patients with CML treated with Imatinib has been collected. Bone marrow biopsy samples are available on >95% of the patients, and pilot studies showed recovery of DNA adequate for our studies from >90% of the cases tested.

To maximize the chances of success, we decided to begin by studying patients for whom frozen cells were available, allowing us access to enough DNA to profile a large number of genes. Initial profiling studies showed a low rate of methylation (<20%) for most of the genes queried. For this reason, we modified this task slightly by designing a case-control study where we intend to compare gene methylation patterns in treatment naïve vs. Imatinib resistant patients. This approach will focus the work on candidates for Imatinib resistance and will maximize the chances of clinical success for this approach. As currently planned, genes showing methylation in at least 20% of Imatinib resistant cases, and at least two-fold more frequent methylation in this group compared to controls will be selected for testing in the larger retrospective and (eventually) prospective series of patients.

We have identified two sets of patients to achieve the initial case-control series discussed above: (1) a set of 80 treatment-naïve patients and (2) a set of 60 Imatinib resistant patients. DNA has been extracted from both sets, and studies are ongoing. We started this analysis by studying methylation of the P15[2] and CDH13[3] genes. The silencing of both of these genes has been implicated as potentially functional in CML. Both genes were studied by bisulfite-pyrosequencing[4], a recently developed quantitative method that provides excellent reproducibility and is therefore ideal for the task. These studies are ongoing. Preliminary data, however, suggests that neither CDH13 nor p15 will fit the criteria defined above to proceed to further testing. Thus, p15 methylation in Imatinib resistant CML had a median of only 4.1%, and only 3 out of 27 cases (11%) had methylation greater than 10% density (required in our previous data to achieve silencing). CDH13 was less frequently methylated than p15.

Thus, to summarize progress for this task, we have successfully identified and retrieved samples from patients with CML treatment naïve or resistant to Imatinib, have extracted DNA from those

and initiated methylation studies. A preliminary screening in the setting of a case-control study was added to the tasks to improve efficiency of the process, and we have already ruled-out the possibilities of involvement of CDH13 and p15 silencing in Imatinib resistance. Studies of other genes are ongoing using a similar approach.

Progress on the related tasks 2 and 3 is jointly summarized below.

Consistent with the stated hypotheses of the grant, we have been testing the activity of decitabine as a single agent and in combination with Imatinib mesylate in the treatment of CML (all phases) resistant to or intolerant of Imatinib. This was achieved through clinical trials at MD Anderson. Initially, a phase I study of decitabine at low doses in all hematologic malignancies was conducted. This study showed optimal clinically activity at relatively low doses and was recently published[5]. Follow-up studies specifically in CML that have so far enrolled 35 patients for the single agent studies and 24 patients for the combined decitabine/Imatinib studies. The clinical treatment of the patients was supported by parallel funding sources. However, all the translational studies that are key to understanding the clinical results were conducted with support from the present DOD grant.

The preliminary clinical results of these studies are summarized in tables 1 and 2.

Table 1: Responses to single agent decitabine in Imatinib refractory or intolerant CML. CHR complete hematologic response. PHR partial hematologic response. HI hematologic improvement. ED early death.

CML phase	Chronic	Acc.	Blastic	Total	
N	11	16	6	33	
% Hematologic Responses					
CHR	45	25	17	30	
PHR	27	13	17	18	61%
HI		19	17	12	
ED			17	3	
% Cytogenetic Responses					
Complete	18	0	0	6	
Major	0	13	17	9	39%
Minor	36	19	17	24	

Median response duration was 3 months, ranging from 2 to 13+ months, and responses are ongoing in 4 patients. The only common grade 3 or 4 toxicities observed were related to myelosuppression. Neutropenic fever developed in 18 patients (51%), in 27/115 (23%) courses of therapy. There were two deaths on study, both related to thrombocytopenia and hemorrhage.

Table 2: Responses to the combination of decitabine and Imatinib in Imatinib refractory or intolerant CML.

CML PHASE	#	Hematologic Response		Cytogenetic Response	
		Complete	Partial	Complete	Partial
Accelerated	10	3	1	1	0
Blast	6	1	2	1	2
Total	16	4 (25%)	3 (19%)	2 (13%)	2 (13%)
		7 (44%)		4 (25%)	

As part of the clinical studies, samples have been collected from patients before therapy, at days 5 and 12 after therapy and at recovery of counts (typically days 30-50). These samples are being used to study the effects of decitabine on methylation in CML, as detailed in the original grant application.

We have extensively studied DNA methylation (global and gene specific) with reference to dose and response from patients on early clinical trials, as well as our recent phase I study. These results are detailed in a manuscript submitted for publication and attached to the progress report. Essentially, our findings were (1) dose-dependent decrease in methylation, with a plateau at around 200 mg/m2 cumulative dose, (2) relationship between hypomethylation and response in AML but not CML, (3) significant hypomethylation of p15 (but no correlation with response) and (4) much less significant hypomethylation of normally methylated genes (e.g. H19).

Analysis of the more recent CML studies is now ongoing. Initially, we are focusing on global DNA methylation measurement using an assay of repetitive element methylation recently described by this laboratory[6]. Thus, we used bisulfite treatment followed by PCR using primers specific for LINE elements, and analysis of methylation by pyrosequencing. LINE methylation had a mean +/- standard error of the mean (SEM) of 71.2+/-1.2% prior to therapy (range 56.5-85.8). Methylation decreased to 60.1+/-1.44% after 1 week, 49.7+/-2.9 after 2 weeks, 50.1+/-2.7% after 3 weeks and returned to 67.2+/-2.1% at recovery of counts (median, 46 days). The dynamics of hypomethylation following decitabine (decrease of about 10% at week 1, 10-20% at weeks 2 and 3 and recovery by week 6) were similar after cycle 2 and cycles >2, with no evidence of cumulative hypomethylation or development of resistance to the hypomethylating effect of decitabine. Figure 1 summarizes these results and show patient-specific examples.

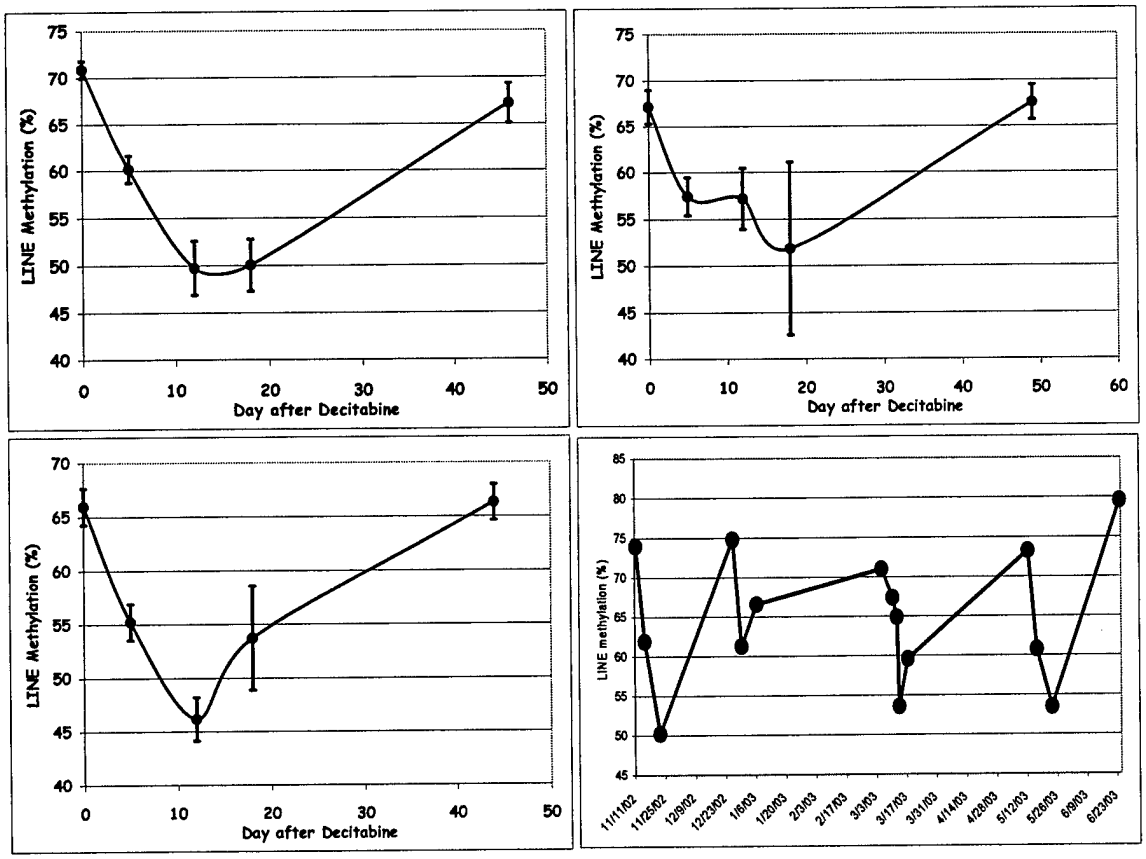

Figure 1: LINE hypomethylation following decitabine therapy in CML (aggregate results from all patients analyzed). Top left: cycle 1. Top right: cycle 2. Bottom left: cycle > 2. Bottom right: example of a single patient receiving four cycles of therapy over 32 weeks.

LINE methylation at the end of week 1 did not correlate with subsequent responses. However, the degree of LINE hypomethylation at the end of therapy (day 12) was paradoxically higher in patients who did not subsequently respond to therapy. Thus, the absolute decrease in methylation was 17.7% vs. 28.6% in responders vs. non-responders ($p=0.009$) and the relative decrease in methylation was 25.0% vs. 39.5% responders vs. non-responders ($p=0.016$). These results are consistent with a cell death mechanism of response, whereby the most hypomethylated cells die rapidly in patients sensitive to therapy, while cells resistant to decitabine can withstand higher degrees of hypomethylation without cell death. Consistent with this concept, in patients who responded and subsequently lost their response while on therapy, the degree of hypomethylation achieved in the cycle immediately prior to relapse was similar or higher than that achieved in prior cycles, indicating that decitabine resistance in this population is not pharmacologic, but likely biologic resistance to hypomethylation-induced cell death.

Current ongoing studies include (1) analysis of methylation of other genes in this patient group (e.g. p15), (2) analysis of gene expression patterns after decitabine therapy, (3) analysis of samples from patients on the combined decitabine and Imatinib study and (4) additional analyses

of methylation patterns at relapse, with particular emphasis on potential mechanisms of resistance to decitabine.

Key research accomplishments

- Identified samples from patients with CML suitable for analysis of mechanisms of resistance to imatinib
- Ruled out methylation of P15 and CDH13 as major causes of imatinib resistance in CML
- Analyzed samples from patients treated with decitabine in a phase 1 study and showed dose dependent hypomethylation in-vivo
- Analyzed samples from patients with imatinib resistant CML treated with decitabine, confirmed hypomethylation in-vivo, found correlations between degree of methylation at 10 days after therapy and lack of response and found similar hypomethylation at the development of resistance to decitabine in CML, suggesting a non-pharmacologic mechanism of resistance
- Collected samples from patients with imatinib resistant CML treated with a combination of imatinib and decitabine

Reportable outcomes

Manuscript submitted (attached in the appendix).

Title: DNA Methylation Changes After Decitabine Therapy In Patients With Leukemia
Authors: Allen S. Yang, Ketan D. Doshi, Sang-Woon Choi, Joel B. Mason, Rajan K. Mannari, Vazganush Gharybian, Rene Luna, Asif Rashid, Lanlan Shen, Marcos R.H. Estecio, Hagop M. Kantarjian, Guillermo Garcia-Manero, Jean-Pierre J. Issa.

Conclusions

In progress to date, we have identified samples from patients with CML prior to Imatinib therapy, as well as from patients with established resistance to Imatinib. Bisulfite based analysis identified methylation of p15 and CDH13 in subsets of patients but ruled these genes out as major causes of resistance. In parallel, clinical trials of decitabine have shown activity as single agent and when combined with Imatinib in CML resistant to Imatinib. Analysis of samples from patients on trial showed hypomethylation after therapy. Hypomethylation dynamics suggest that decitabine leads to CML cell death 5-10 days after treatment and suggest that resistance to decitabine is not pharmacologic. These studies are ongoing to clarify the role of methylation in the pathogenesis and therapy of Imatinib resistant CML. "So what": (1) Methylation analysis does not yet help in predicting imatinib resistance in CML but studies are ongoing; (2) The hypomethylating drug decitabine has clinical activity in imatinib resistant CML, and analysis of CML samples after therapy may predict response to this agent.

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Appendix

1. Yang AS, Doshi KD, Choi S-W, Mason JB, Mannari RK, Gharybian V, Luna R, Rashid A, Shen L, Estecio M, Kantarjian HM, Garcia-Manero G, Issa JPJ. DNA Methylation Changes After Decitabine Therapy In Patients With Leukemia, Submitted.
2. Protocol LAB04-0035, "Epigenetic silencing and resistance to imatinib mesylate in CML"
3. Protocol LAB00-200, "Study of DNA Methylation in Acute Lymphoblastic Luekemia"
4. Protocol LAB01-423, "Use of Stored Samples from Patients with Leukemia and Myelodysplastic Syndromes to Study DNA-Methylation"
5. Protocol LAB00-271, "Use of Paraffin-Embedded Archival Samples from Patients with Leukemia and Myelodysplastic Syndromes to Study DNA-Methylation"

DNA Methylation Changes After Decitabine Therapy In Patients With Leukemia

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Abstract

5-aza-2'-deoxycytidine (decitabine) is postulated to have clinical activity in myeloid leukemias via its ability to inhibit DNA methylation. To study this, we examined DNA methylation in patients with leukemia treated with decitabine. Five days after treatment, total genomic 5-methylcytosine/cytosine decreased on average by 14% (from 4.3% to 3.7%), while methylation of repetitive DNA elements showed a mean decrease of 9% and 16% for Alu and LINE elements respectively. Methylation decreased linearly with increasing doses between 5 and 20 mg/m^2/day (R=0.88, p=0.05), but showed a plateau above that. Hypomethylation correlated with response in patients with AML treated with low doses (5-20mg/m^2/day) but patients with CML treated with high doses (100 to 180 mg/m^2/day) showed no such correlation. Aberrant methylation of p15 (>10%) was found in 24% of patients and 80% of these showed a decrease by at least one third, but this did not correlate with response. The imprinted gene H19 showed little change in methylation after decitabine. In conclusion, we show dose dependent hypomethylation after decitabine at low doses. Increasing the dose, which had previously been shown to result in a reduced response rate, was not accompanied by further hypomethylation. At low doses, hypomethylation correlated with response in AML.

Introduction

5-aza-2'-deoxycytidine (decitabine) is a pyrimidine analog first synthesized almost 40 years ago. Early clinical trials showed that decitabine had consistent activity in patients with myeloid leukemia. There is considerable experience in the use of decitabine and a similar drug, 5-azacytidine, in several clinical trials in patients with chronic myelogenous leukemia (CML), acute myeloid (AML) and myelodysplastic syndrome (MDS) (Glover, Leyland-Jones et al. 1987; Zagonel, Lo Re et al. 1993; Santini, Kantarjian et al. 2001; Gryn, Zeigler et al. 2002).

Decitabine once incorporated into DNA in place of cytosine can covalently trap DNA methyltransferase to DNA causing irreversible inhibition of the enzyme(Christman 2002). This covalent enzyme-DNA adduct can be cytotoxic at high doses. By contrast low dose schedules of decitabine are believed to capitalize on the drug's ability to inhibit DNA methylation and to reactivate gene expression (Jones and Taylor 1980). Recent efforts have focused on administering lower doses of decitabine to minimize toxicity and to exploit the unique property of decitabine to inhibit DNA methylation (Chitambar, Libnoch et al. 1991; Wijermans, Lubbert et al. 2000; Lubbert, Wijermans et al. 2001; Issa, Garcia-Manero et al. 2004). Interestingly in a phase I study of low-dose decitabine, 15mg/m^2 given over 10 days, which is significantly lower than the maximally tolerated dose, seemed to be the optimal clinically effective dose with a response rate of about 50%(Issa, Garcia-Manero et al. 2004).

Methylation of cytosine residues in gene promoters is associated with gene silencing, and aberrant DNA methylation patterns are associated with tumorigenesis. It is believed that altered DNA methylation patterns and epigenetics play a crucial role in cancer and differentiation(Jones and Baylin 2002; Karpf and Jones 2002; Herman and Baylin 2003). Aberrant DNA methylation patterns of multiple genes have been described in leukemia as well

as other tumor types(Issa, Kantarjian et al. 1999; Toyota, Kopecky et al. 2001; Garcia-Manero, Bueso-Ramos et al. 2002; Paz, Fraga et al. 2003). Altered methylation of certain gene loci is associated with prognosis, and methylation changes may define different subtypes of leukemia. Decitabine has been shown to reverse the aberrant hypermethylation of the p15 gene in some patients with AML and MDS(Daskalakis, Nguyen et al. 2002; Issa, Garcia-Manero et al. 2004).

In this study, we examined global and gene specific DNA methylation changes in the peripheral blood of patients with leukemia treated with decitabine. We found a dose dependent hypomethylation response that remarkably parallels in vitro studies, and a relationship between hypomethylation dependent responses at low but not high doses of decitabine.

Materials and Methods

Patient Samples

Samples were collected from patients treated as part of two clinical studies using decitabine as a single agent. The first study was a phase II study in CML conducted from 1993 to 2000 (prior to the availability of imatinib mesylate) and treated patients with 50-90mg/m^2 of decitabine twice daily for 5 consecutive days (See Figure 1)(Kantarjian, O'Brien et al. 2003). Pre and post-treatment samples were available from 18 patients in this study. The second study was a phase I study which attempted to capitalize on the demethylating properties of decitabine by using a lower dose of decitabine, 5-20mg/m^2 once daily, for a longer period of time, 10 days over a two week period (See Figure 1)(Issa, Garcia-Manero et al. 2004). Samples were available from 27 patients in this study. Peripheral blood samples were collected prior to, during and after treatment. Donation of blood samples for laboratory studies was voluntary and patients gave informed consent for sample collection according to institutional guidelines.

DNA Isolation and Bisulfite Treatment

DNA was isolated from peripheral blood samples after ficoll separation of mononuclear cells using standard phenol-chloroform extraction methods. DNA was treated with bisulfite, which selectively deaminates cytosine but not 5-methylcytosine to uracil. This leads to a primary sequence change as unmethylated cytosines are converted to uracil and then thymidine after PCR, however, 5-methylcytosine is not converted by bisulfite and remains as a cytosine after PCR(Clark, Harrison et al. 1994). This primary sequence change can be quantitated using direct sequencing, restriction digestion or pyrosequencing. In brief, 1.5 μg of DNA was denatured in 50

µl of 0.2 M NaOH for 10 minutes at 37°C. Then 30 µl of freshly prepared 10mM hydroquinone (Sigma) and 520 µl of 3 M Sodium Bisulfite (Sigma) at pH= 5.0 were added and mixed. The samples were overlayed with mineral oil to prevent evaporation and incubated at 50°C for 16 hours. Bisulfite treated DNA was isolated using the Wizard DNA Clean-Up System (Promega). DNA was eluted by 50 µl of warm water and 5.5 µl of 3 M NaOH were added for 5 minutes. DNA was ethanol precipitated with glycogen as a carrier and resuspended in 20 µl water. Bisulfite treated DNA was stored at -20°C until ready for PCR amplification and analysis by cloning followed by direct sequencing, restriction digestion (COBRA), or pyrosequencing.

Analysis of Alu and LINE-1 repetitive element metylation

A previously described COBRA analysis of Alu repetitive DNA elements was used as a surrogate for global DNA methylation changes(Yang, Estecio et al. 2004). PCR primers directed towards an Alu consensus sequence were used in a low stringency PCR reaction to amplify a pool of approximately 15,000 Alu elements. Then a restriction digest was used to distinguish methylated from unmethylated sequences. The digested PCR products were then run on an Agilent Biosystems DNA analyzer to quantitate the cut (methylated) and uncut (methylated) DNA.

The LINE-1 assay was based on a similar principle to the Alu element COBRA assay, but used non-selective PCR of Long Interspersed Nucleotide Elements and pyrosequencing to quantitate methylation. A 50 µl PCR was carried out in 60 mM Tris-HCl pH=8.8, 15mM Ammonium Sulfate, 0.5 mM $MgCl_2$, 1mM dNTP mix, and 1 unit of Taq polymerase. The PCR primers used: 10 pmol of 5'-TTTTTTGAGTTAGGTGTGGG-3', 1pmol of 5'-GGGACACCGCTGATCGTTTATCTCACTAAAAAATACCAAACAA-3', and 10 pmol of a

universal biotinylated primer 5'-GGGACACCGCTGATCGTTTA-3'. PCR cycling conditions were 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds for 35 cycles. The PCR product was purified and quantitated using the PSQ HS 96 Pyrosequencing System (Pyrosequencing, Inc.; Westborough, MA). The sequencing primer for pyrosequencing was 5'-GGGTGGGAGTGAT-3'.

Combined Bisulfite Restriction Analysis (COBRA) of HoxA5 and H19

COBRA methylation(Xiong and Laird 1997) analysis was performed for two gene loci. HoxA5 is a gene heavily methylated in normal and leukemic cells(Garcia-Manero, Daniels et al. 2001). H19 is an imprinted gene locus that is methylated on the paternal chromosome, and unmethylated on the maternal chromosome. See table 2 for PCR primer sequences, PCR cycling conditions and restriction enzymes used. Digested PCR products were quantitated by densitometry after separation by polyacrylamide gel electrophoresis.

Methylation analysis of the p15 gene

The p15 tumor suppressor gene, which is normally unmethylated or lightly methylated, but becomes aberrantly methylated in cancer, was also examined. Methylation of the p15 tumor suppressor gene was difficult to analyze consistently by COBRA due to the relatively low level of aberrant methylation at this locus. Therefore pyrosequencing was used to analyze methylation of the p15 gene. A 50 µl PCR was carried out in 60 mM Tris-HCl pH=8.8, 15mM Ammonium Sulfate, 2 mM $MgCl_2$, 1mM dNTP mix, and 1 unit of Taq polymerase. PCR primers used were 10 pmol of 5'-GTTTTTTTTTAGAAGTAATTTA-3', 1 pmol of 5'-GGACACCGCTGATCGTTTATCCTTCTACGACTTAAAACC-3', and 10 pmol of a universal

biotinylated primer 5'-GGGACACCGCTGATCGTTTA-3'. PCR cycling conditions were melting temperature of 95°C for 30 seconds, annealing for 45 seconds at a temperature of 50°C for 3 cycles, 48 °C for 4 cycles, 46 °C for 4 cycles, 44 °C for 4 cycles, 42 °C for 43 cycles, and extension temperature of 72°C for 45 seconds. The sequencing primer for pyrosequencing was 5'- TTTTTAGAAGTAATTTAGG -3'

For selected cases PCR products were cloned using the TOPO-TA cloning kit (Invitrogen) per the manufacturers protocol, and multiple different clones were sequenced. Mini-preps were prepared using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The M.D. Anderson Cancer Center Sequencing Core Facility performed the DNA sequencing.

Liquid Chromatography Mass Spectroscopy

Total 5-methylcytosine content was quantitated in genomic DNA using a previously described highly specific assay using liquid chromatography coupled with mass spectrometry (LC)/MS (Friso, Choi et al. 2002). In brief 0.5 μg of genomic DNA was hydrolyzed by sequential digestion with three enzymes, nuclease P1 (Roche Molecular Biochemicals, Mannheim, Germany), venom phosphodiesteraseI (Sigma, St. Louis, MO) and alkaline phosphatase (Sigma, St. Louis, MO). The hydrolyzed DNA solution was directly delivered onto the analytical column (Supelco, Bellefonte, PA) in isocratic mode. This allowed the separation of the four DNA bases as well as the identification of 5-methylcytosine. Identification of cytosine and 5-methylcytosine was obtained by mass spectrometry analysis of chromatographic peaks. The isotopomers $^{15}N_3$ 2'-deoxycytidine and methyl-D3, ring-6-D1 5-methyl-2'-deoxycytidine (Cambridge Isotope Laboratories, Cambridge, MA) were used as internal standards allowing the quantitation of absolute amounts of the deoxycytidine and methylated cytosine residues in

genomic DNA. DNA methylation status is presented as percentage of 5-methylcytosine in total cytosine.

p53 Sequencing

Mutation analysis of the p53 gene was performed by direct sequencing of exons 5 through 9 of the p53 gene. Genomic DNA was amplified using PCR (see Table 2), and the PCR product was purified. DNA sequencing was performed using primers used for PCR amplification with the ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems, CA, USA) and the ABI PRISM™ 3730 DNA Analyzer (Applied Biosystems, CA, USA). Samples containing mutations were confirmed by reamplification of genomic DNA and sequencing.

Statistical Methods

Microsoft Excel was used to analyze the data. To determine the relationship of DNA methylation changes before and after treatment with decitabine a two-sided t-test was used to compare methylation levels before and after treatment. To determine the relationship of DNA methylation changes and response to decitabine therapy a two-sided t-test was used to compare methylation levels of the patients whose disease responded to the patients whose disease did not respond. Two-sided p values are reported and $p<0.05$ was considered significant.

Results

Decitabine induces hypomethylation in vivo

In order to see if decitabine could inhibit methylation over the entire genome we quantitatively measured the methylation of Alu repetitive elements, LINE repetitive elements and total 5-methylcytosine content of patient samples collected before, during and after treatment with decitabine.

Bisulfite treatment of DNA followed by a PCR reaction that amplifies a pool of Alu or LINE repetitive elements can be used as a surrogate marker for genome wide DNA methylation changes(Yang, Estecio et al. 2004). Examination of repetitive elements seemed to have several advantages over gene specific loci examined, because repetitive elements are very abundant and usually heavily methylated in the genome. Thus PCR reactions were very robust and could be performed on very small amounts of DNA. In addition very small decreases in methylation could be detected when using a sensitive quantitation system (Figure 2A).

Methylation of Alu repetitive elements in peripheral blood samples of leukemia patients ranged from 23.6% to 29.3% with a mean methylation of 26.9% (standard error of the mean [SEM]=0.27%) prior to treatment with decitabine (Figure2B). As previously reported, this assay underestimates the amount of methylation because mutations of Alu elements will appear as unmethylated CpG sites (Yang, Estecio et al. 2004). Obvious decreases in methylation could be detected after decitabine treatment with a decrease in mean methylation to 26.0% (SEM= 0.35%, Range 18.0 to 29.3%, p=0.036) by day 2 to 4 and to 24.5% (SEM=0.51%, Range 16.9% to 29.1%, p<0.001) by day 5 to 8 in both studies combined. Samples were not collected from days 9 to 14 for the high dose study, but in the low dose study DNA methylation did not significantly

decrease beyond day 8 with a mean methylation plateau of 25.1% (SEM=0.47, Range 19.0% to 28.2%, p=0.002) on days 9 to 14 (Figure 2B). The mean decrease in absolute Alu methylation was 2.3%, which corresponds to a relative decrease of 9% (Range 0 to 31.5%). The maximum relative methylation decrease was 31.5% in one patient.

Similar results were obtained for LINE methylation. Baseline methylation of LINE elements prior to treatment was 70.2% (SEM=0.90, Range 55.1% to 77.7%). After decitabine treatment LINE methylation decreased to 65.0% (SEM=1.65, Range 26.2% to 86.0%, p=0.008) by day 2 to 4 and 59.2% (SEM=1.90, Range 35.0% to 76.2%, p<0.001) by day 5 to 8 in both studies combined. Again a plateau was seen with mean methylation 59.9% (SEM=2.11, Range 35.7% to 74.4%, p<0.001) on days 9 to 14 of decitabine treatment (Figure 3B).

Finally these data were confirmed by direct measurement of 5-methylcytosine in the genome using liquid chromatography-mass spectroscopy (LC-MS). This required more DNA and data was obtained from only 103 of the original 143 samples. Prior to treatment 5-methylcytosine made up on average 4.3% of cytosine in the genome of patients. After decitabine treatment, mean 5-methylcytosine decreased to 4.1% (SEM=0.12%, Range 2.9% to 5.4%, p=0.10) by day 2 to 4 and 3.7% (SEM=0.19%, Range 1.7% to 5.3%, p=0.009) by day 5 to 8 in both studies combined. In the low dose study mean 5-methylcytosine content decreased to 3.8% (SEM=0.11%, Range 2.5% to 4.8%, p=0.003) by days 9 to 14 (Figure 4).

Dose Dependence of Decitabine inhibition of DNA Methylation

To examine the dose dependence of decitabine-induced hypomethylation, we pooled the patients treated in both studies and compared day 5-6 values across different doses. Only one patient sample was available at the 20mg/m^2/day dose level for days 5 to 6. Mean methylation of

Alu elements prior to treatment was 26.8% and by day 5 to 6 mean methylation decreased to 25.7%, 26.5% 24.5%, 23.4% and 23.9% for 5, 10, 15, 20 and 100mg/m^2/day of decitabine respectively (Figure 5A). Similar results were obtained for LINE repetitive elements with mean LINE methylation decreasing from 70.2% to 71.4%, 62.8%, 60.1%, 59.4% and 55.7% for 5, 10, 15, 20 and 100mg/m^2/day of decitabine respectively (Figure 5B). Thus, there was a dose-dependent linear decrease in methylation at low doses of 5 to 20 mg/m^2/day (R= 0.88, p= 0.046) with no significant increase in hypomethylation beyond 20mg/m^2/day in the Alu and LINE assays suggesting a plateau effect. Assessment of 5-methylcytosine by liquid chromatography did show greater decreases at higher doses but a plateau phenomenon was not seen (Figure 5C). It is unclear whether this is due to fewer samples being studied, differences in the assays, or differences in the methylation dynamics of repetitive elements compared to the entire genome.

Hypomethylation and Response to Decitabine

A critical question is whether decitabine has clinical activity in cancer through its ability to induce hypomethylation. We initially examined this issue using the Alu assay. In the high dose study comprising mainly CML patients, both responders and non-responders to decitabine showed a decrease in Alu element methylation (Figure 6A) with no difference between the two groups (Mean decrease of 1.3% in responders and 4.1% in non-responders, p=0.23).

By contrast, in the low dose study (mostly AML patients) decreases in methylation were seen in both non-responders and responders, but the responders showed a statistically significant larger decrease by days 5-8 and days 9-14 (day 5-8: 2.6%, p=0.04; day 9-14: 3.4%, p=0.02) when compared to non-responders (1.1% days 5-8 and 1.2% days 9 to 14) (Figure 6B).

Similar trends were observed using analysis of LINE element methylation and global methylation, with greater decreases in methylation being observed in the non-responders in the high dose study (Figure 6C and E), and in the low dose study responders again showed a greater decrease in methylation when compared to the non-responders (Figure 6D and F). These differences however did not reach statistical significance.

Methylation of HoxA5 and H19 in Patients Treated with Decitabine

In addition to studying global changes in DNA methylation we examined two gene specific loci that are normally methylated. HoxA5 is a gene that is normally hypermethylated in normal peripheral blood mononuclear cells (Figure 7A), and H19 is an imprinted gene that is normally methylated only on the paternal allele and unmethylated in the maternal allele (Figure 7C).

The HoxA5 locus was heavily methylated in all patients examined (Mean 56.5% (SD=25.7%), with a range of 11.0 to 95%) (Figure 7 B). The mean methylation of HoxA5 did not change significantly in either the high dose study or the low dose study. Mean methylation in the high dose study at baseline was 58.8% (SEM= 4.3%), 53.3% (SEM= 4.1%, p=0.37) by day 2 to 4 of treatment, and 55.1% (SEM= 5.1%, p=0.52) by day 5 to 8 of treatment. Overall the patients in the low dose study showed less HoxA5 methylation at baseline 38.2% (SEM= 4.3%), decreasing to 34.7% (SEM=5.2%, p=0.60) on day 2 to 4, 40.0% (SEM=8.5%,p=0.85) on day 5 to 8, and 36.5% (SEM= 5.6%, p=0.81) on days 9 to 14. Taking the maximum change in methylation observed, overall 38% (9/24) of patients analyzed showed an absolute decrease in methylation greater than 10% (Range –31.0 to 43.1%, SD=14.8%). Only one of 24 patients (4%)

showed an increase of methylation >10%. The majority of patients (58%, 14/24) showed no change or a change in methylation of less than 10%.

H19, an imprinted gene that is only methylated on the paternal allele, was found to have around 46.9% (Range 30-58%, SD=7.7%) methylation prior to treatment with decitabine (Figure 7D). This is close to the expected 50% methylation for an imprinted gene, and baseline methylation was consistent between the two studies. A decrease in absolute methylation of greater than 10% could be detected in only 12% (4/33) of patients treated with decitabine with a mean decrease in methylation of only 1.1% (Range –16.9 to 32.9%, SD=13.6%). An increase in methylation of greater than 10% was seen 12% (4/33), and the remaining 76% (25/33) patients showed changes less than 10%. Although methylation changes were observed for both HoxA5 and H19 after decitabine treatment, a dose dependent response could not be shown, and there was no correlation between response and hypomethylation and at either locus.

Methylation of the p15 Gene in Patients Treated with Decitabine

We quantitated methylation of the p15 gene using bisulfite pyrosequencing. Methylation at the start of treatment ranged broadly from 0 to 74% with a mean methylation of 11.1% (SEM= 4.5%) and 12.9% (SEM=3.7%) for the high dose and low dose studies respectively (Figure 8B). Combining both studies the median amount of p15 methylation was only 4.0% demonstrating that aberrant methylation of p15 was infrequent. Substantial methylation (>10%) could be seen in 27% (10/37) of patients at the start of treatment. Overall, decitabine decreased mean p15 methylation by 7% (Range 0 to 42.9%). Of the 10 patients who had greater than 10% methylation prior to treatment (Mean 36.0%, Range 13.9 to 73.8%) the average relative decrease in methylation was 62.6% (Range –12% to 100%). To confirm these results, bisulfite sequencing

was performed on samples from one patient with a high level of methylation. The data shown in figure 8A shows directly that methylation can be inhibited from days 0 to 5 of treatment and are in general agreement with the pyrosequencing data obtained for this patient.

Although methylation changes could be shown for p15 during treatment, no correlation between induction of hypomethylation and decitabine dose or response could be found. However, p15 methylation status prior to treatment paradoxically predicted a lower response to the drug. Mean methylation of patients who did not respond to p15 was 15.9% compared to 5.0% for patients who did respond ($p=0.02$). No patient who had greater than 25% methylation of p15 prior to treatment responded to decitabine, and 85% (11/13) patients who had a CR or PR following decitabine treatment had <10% methylation of p15 at baseline. There were no pretreatment differences in Alu, LINE, HoxA5 or H19 methylation levels in responders compared to non-responders (data not shown).

p53 Mutations and Response to Decitabine

Previous reports suggested that the absence of p53 is critical for the induction of apoptosis by decitabine at relatively high doses (Jackson-Grusby, Beard et al. 2001; Nieto, Samper et al. 2004). In order to determine the clinical relevance of this finding we sequenced exons 5 through 9 of p53 in patients treated with decitabine. Exons 5 through 9 of p53 are conserved domains in which the majority of p53 mutations occur(Harris 1996). We identified only three patients of 41 studied with p53 mutations that change the amino acid composition. This low frequency of mutation (7%) is consistent with previous reports in hematological malignancies (Peller and Rotter 2003). Of the three patients with p53 mutations all had AML,

one had a complete response and two did not respond to decitabine. Therefore no correlation was found between p53 mutation and response to decitabine.

Discussion

We have examined peripheral blood samples from leukemia patients treated in two clinical trials using decitabine. The first trial enrolled predominately CML patients and used cytotoxic doses of decitabine for a short period of time. The second study treated mostly AML patients with a low dose of decitabine for a longer period of time in order to take advantage of decitabine's ability to inhibit DNA methylation. We have shown using multiple assays that methylation clearly decreases both globally and in a gene specific manner following decitabine treatment. Global changes in DNA methylation were dose dependent with a plateau, and correlated with response to decitabine at low doses but not high doses.

We chose to study peripheral blood specimens in this study due to the ease of collection and ability to follow frequent serial samples. Leukemic blasts were not purified from the samples and the DNA isolated in our study is a mixture of normal appearing leukocytes and blasts. Our data reflects methylation changes in a mixture of blasts and normal appearing leukocytes, and it is possible that the methylation changes observed are occurring preferentially in one population. However, previous work has shown that in patients with leukemia normal appearing cells have cytogenetic and methylation changes identical to those of the leukemic clone(Baylin, Fearon et al. 1987). In addition, the therapeutic effect of decitabine is delayed with responses occurring several weeks after treatment without significant changes in the peripheral blood(Kantarjian, O'Brien et al. 2003). In the short period from which samples were collected the white blood cell count (WBC) and blast percentage did not change significantly. The mean WBC count, for all the patients in both studies, decreased slightly from 13.8 (SEM=3.3) to 10.7 thousand (SEM=2.5) on days 5 to 8. The mean blast percentage slightly increased from 20.7%

(SEM=4.5%) to 25.4% (SEM=6.2%) on days 5 to 8 of treatment. Therefore samples collected during a course of treatment were very similar in terms of WBC and percentage of leukemic blasts for any given patient. It is also possible that decitabine treatment leads to selective toxicity of hypermethylated cells and a selection and expansion of hypomethylated clones instead of induction of true hypomethylation. In order to account for this possibility the study was designed to assay frequent serial samples from very early (days) after the start treatment and not after completing therapy (weeks). This minimizes the possibility that a selection phenomenon is being observed, and true inhibition of methylation is being observed. For these reasons, we believe that our data truly reflect dynamic changes in methylation in leukemic cells.

We observed a linear decrease in methylation with increasing decitabine doses from 5 to 20 mg/m^2/day, and interestingly in DNA repetitive elements there was no significant decrease in methylation at higher doses. The mechanism of this plateau warrants further study. It may reflect a simple pharmacokinetic phenomenon in which the metabolism and incorporation of decitabine into DNA is saturated at higher doses. A given level of hypomethylation could conceivably lead to inhibition of further decitabine incorporation, or this threshold level of hypomethylation may lead to cell death and therefore a selection of cells with a fixed "maximum" hypomethylation. Alternatively, this dose plateau may reflect the rapid induction of remethylation past a given threshold, and in-vitro work provides evidence for such remethylation(Bender, Gonzalgo et al. 1999). Thus higher doses of decitabine may add toxicity, but no further ability to induce DNA hypomethylation. This biochemical evidence supports the use of low dose decitabine in clinical practice.

The finding that decreases in global methylation correlate with clinical benefit of decitabine only in the low dose study may be attributable to the disease, predominately CML in

the high dose study versus AML in the low dose study. A more attractive possibility is that the actual dosing regimens may be crucial to how decitabine has clinical activity. In the high dose study decitabine was given at high doses, and may act as a cytotoxic pyrimidine analog, whereas in the low dose study decitabine was given at low doses for longer periods of time to take advantage of its demethylating properties. This is in agreement with previous studies in vitro have shown that the ability of decitabine to induce cellular differentiation is optimal at lower doses (Taylor and Jones 1979). Although not definitive proof, it adds further credence to DNA methylation inhibition as decitabine's therapeutic mechanism of action.

Comparison of all the loci examined showed variable decreases in mean methylation after treatment with decitabine. Although the methods used to measure DNA methylation varied, the relative decrease of methylation was LINE (15.6%)> total 5-methylcytosine (14.1%)> Alu elements (9.0%)> p15 (4.0%)> HoxA5 & H19 (No Change). Interestingly the mean decrease of p15 methylation in patients who were hypermethylated at that gene was 62.6% showing that genes aberrantly methylated in cancer may be particularly susceptible to the drug. The mechanisms of this gene specific effect, and whether it contributes to the therapeutic index remains unknown(Farinha, Shaker et al. 2004). Nevertheless, it is reassuring that normally methylated genes do not show large decreases in methylation, which had been a concern with this therapy(Lengauer, Kinzler et al. 1997; Eads, Danenberg et al. 1999; Eden, Gaudet et al. 2003; Gaudet, Hodgson et al. 2003; Yang, Estecio et al. 2003).

Possibly the simplest explanation as to how decitabine has activity in cancer is that it reactivates expression of tumor suppressor genes that have been silenced by aberrant DNA methylation. One would expect tumor suppressor genes, like p15, that are aberrantly methylated and silenced in leukemia are reactivated by treatment with decitabine. We have shown a

preferential susceptibility of the hypermethylated p15 gene to inhibition of methylation by decitabine, which may help explain the cancer selective toxicity of decitabine and therefore in part its therapeutic index. However, methylation of p15 inversely correlated with response with high pretreatment levels correlating with resistance to decitabine treatment. This finding may be due to other confounding factors such as increased bone marrow blasts that correlate with p15 methylation (data not shown). Alternatively, hypomethylation of p15 may not be a crucial target gene for decitabine treatment, and methylation changes of other tumor suppressor genes and genomic loci need to be evaluated. Previous studies have shown that decitabine can induce expression of tumor antigens that could theoretically induce an anti-tumor immune response(Sigalotti, Altomonte et al. 2003). If this were true sequential therapy of decitabine followed immunotherapy could prove valuable.

Our work expands on previous studies showing the ability of decitabine to inhibit DNA methylation in patients. It highlights the difficulty in using gene specific assays to measure DNA methylation changes, but shows that global methylation changes occur and correlate with both dose and response to decitabine. Ironically, we show that patients whose leukemia was hypermethylated at one tumor suppressor gene, p15, were less likely to respond to decitabine. Nevertheless, our work supports in vitro work that lower doses of decitabine are optimal for hypomethylation and encourages pursuit of low dose clinical strategies and the pursuit of other genomic loci in which hypomethylation is induced by decitabine.

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Figure Legends:

Figure 1: Treatment Schema
Patients were treated as part of a Phase II study of decitabine that was given initially at a dose of 90mg/m2 twice daily for 5 consecutive days. This dose was later decreased to 50mg/m^2 twice daily due to toxicity. In this "High" dose study a total dose of 500-900mg/m^2 of decitabine was given over a 5-day period. In the second study, a Phase I strategy of "Low" dose decitabine was used. This study was designed to take advantage of decitabine's hypomethylating properties. Escalating doses of decitabine of 5, 10, 15 and 20 mg/m^2 were given as ten daily doses over a two-week period. The total dose delivered was only 50 to 200mg/m^2 over a 12-day period.

Figure 2: Alu Element Methylation Analysis of Leukemia Patients treated with Decitabine
Methylation of peripheral blood from leukemia patients was assessed for sequential days of decitabine treatment. A pool of Alu elements was amplified by bisulfite-PCR. Methylation was analyzed by restriction digestion with MboI, which cuts only if the original DNA was methylated, followed by quantitation by capillary electrophoresis.
A) Representative data is shown from a leukemia patient treated with decitabine on day 0, 2 and 5 of treatment. B) Mean Alu methylation for leukemia patients in the High dose study (black bars) and Low dose study (grey bars) are shown. Methylation decreases are seen for both studies during treatment. Note that no samples were collected on days 9 to 14 for patients in the high dose study. The standard error of the mean is shown by the error bars.

Figure 3: LINE Element Methylation Analysis of Leukemia Patients treated with Decitabine
Methylation from leukemia patients was assessed for sequential days of decitabine treatment. A pool of LINE elements was amplified by bisulfite-PCR, and quantitated by pyrosequencing. A) Representative data is shown from a leukemia patient treated with decitabine on day 0, 2 and 5 of treatment. The decreasing methylation is indicated by an arrow B) Mean LINE methylation for leukemia patients in the High dose study (black bars) and Low dose study (gray bars) are shown. Methylation decreases are seen for both studies during treatment.

Figure 4: Quantification of Methylation by Liquid Chromatography Mass Spectroscopy
Total 5-methylcytosine was quantitated for leukemia patients treated with decitabine on sequential days of treatment. Methylation was quantitated by digestion of total genomic DNA into single nucleotides and quantification of 5-methylcytosine by LC-Mass Spectroscopy. Total 5-methylcytosine is reported as a percentage of total cytosine measured. Mean methylation for all patients treated on the high dose study (black bars) and low dose study (gray bars) are shown.

Figure 5: Hypomethylation Dose Response of Decitabine
Data from both the low dose (5, 10, 15 and 20 mg/m^2/day) and the high dose (100mg/m2/day) decitabine studies were combined. DNA methylation of Alu elements (A), LINE (B) and 5-methylcytosine by LC-MS(C) at days 5 to 6 was compared to the dose given. The measurement of methylation inhibition in Alu elements (A) and LINE elements (B) showed that approximately 15 to 20 mg/m^2/day appeared to be the optimal demethylating dose with no significant decrease in methylation with higher doses of decitabine. Note only one patient sample was available for

the patients treated at 20mg/m^2/day. Similar data was obtained for quantification of methylation by LC-MS (C), but data was obtained from fewer samples.

Figure 6: Hypomethylation and Response
Hypomethylation of Alu Elements was compared in those patients who responded to Decitabine and those who did not respond to Decitabine for both the High Dose Study (A, C, E) and the Low Dose Study (B, D, F). In the High Dose Study methylation inhibition did not correlate to response, and surprisingly there was a trend for non-responders to have a greater decrease in methylation in Alu elements (A) ($p=0.23$). In the Low Dose Study, methylation inhibition at Alu elements did correlate with response (B) ($p=0.04$ days 5-8 and $p=0.02$ days 9-14). Similar results were obtained for the LINE assay and LC-MS assay with a trend to greater decrease in methylation in the non-responders (C, E) in the high dose study and more methylation inhibition in the responders in the low dose study (D, F) but these trends did not reach statistical significance.

Figure 7: Gene Specific Methylation Analysis of Leukemia Patients treated with Decitabine
Methylation of peripheral blood from leukemia patients was assessed for sequential days of decitabine treatment. Methylation was quantitated by bisulfite-PCR followed by restriction enzyme digestion (COBRA). Analysis of HoxA5 (A) and H19 (C) was quantitated by densitometry of bands on polyacrylamide gel. Representative data is shown, and the arrows indicate methylated bands/peaks. B) Mean methylation of HoxA5 for the high dose study (black bars) and low dose study (gray bars) are shown on sequential days of treatment. D) Mean methylation of H19 for the high dose study (black bars) and low dose study (gray bars) are shown. No trends in decreasing methylation can be shown for the HoxA5 or H19 genes with decitabine treatment.

Figure 8: Methylation changes of p15 induced by Decitabine
(A) Map of the p15 gene with CpG sites shown as hatch marks. The transcription start site is shown by the arrow and the beginning of the coding sequence (CDS) is indicated. The PCR product used for both pyrosequencing and cloning-sequencing is shown. PSQ indicates the bases assayed by pyrosequencing. Direct bisulfite sequencing of a single patient treated with decitabine is shown. Black circles represent methylated CpG sites and white circles represent unmethylated sites. By pyrosequencing, methylation prior to treatment with decitabine was 63% with a decrease to 42% by day 2 and a further decrease to 8% by day 5. Methylation of p15 was then quantitated using bisulfite PCR and pyrosequencing. B) Mean methylation of p15 for the high dose study (black bars) and low dose study (gray bars) are shown.

Table 1: Samples Studied

The majority of patients treated in the High Dose Study were CML patients and the majority of patients treated in the Low Dose Study were AML or high-risk MDS patients. Donation of peripheral blood for laboratory analysis was voluntary, and only some of the patients enrolled on the two studies participated. A total of 143 samples were collected for analysis (52 in the High Dose Study and 91 in the Low Dose Study).

		Diagnosis		Treatment response		
Study	Patient #	AML/MDS	CML/CMML	CR/PR	No Response	Response Rate
High Dose Study	18	3	15	7	11	39%
Low Dose Study	27	24	3	7	17	26%
Total	45	27	18	14	28	31%

Table 2: Genes Studied and PCR Conditions

Gene	Primers	Restriction Enzyme	Annealing Temperatures (no. of cycles)
HoxA5	5'-TAATGGGTTGTAATTTTAATT-3' 5'-TAATAACCTCTAAAAATAAACTC-3'	RsaI BstUI	53(3), 52(4), 50(5), 48(29)
H19	5'-GGAGGGTTTTGTTTTGATTGGT-3' 5'-CAACACCCCATCTTCCCCTAAT-3'	DpnII	67(2), 65(3), 63(4), 61(28)
Alu	5'-GATCTTTTTATTAAAAATATAAAAATTAGT-3' 5'- GATCCCAAACTAAAATACAATAA-3'	MboI	43(27)
LINE	5'-TTTTTTGAGTTAGGTGTGGG-3' 5'GGGACACCGCTGATCGTTTATCTCACTAAAAAA TACCAAACAA-3',	Pyrosequencing	50(35)
P15	5'-GTTTTTTTTTAGAAGTAATTTA-3' 5'GGACACCGCTGATCGTTTATCCTTCTACGACTT AAAACC-3'	Pyrosequencing	50(3), 48(4), 46(4), 44(4), 42(43)
P53 Exon 5	5'-GACTTTCAACTCTGTCTCC-3' 5'-GAGCAATCAGTGAGGAATC-3'	Direct Sequencing	55(45)
P53 Exon 6	5'-TCCCCAGGCCTCTGATTCC-3' 5'-TGACAACCACCCTTAACCC-3',	Direct Sequencing	61(45)
P53 Exon 7	5'-CAAGGCGCACTGGCCTCATC-3' 5'- CACAGCAGGCCAGTGTGCAG-3'	Direct Sequencing	65(45)
P53 Exon 8	5'-GATTTCCTTACTGCCTCTTGC-3' 5'-GTGAATCTGAGGCATAACTGC-3'	Direct Sequencing	61(45)
P53 Exon 9	5'-GCCTCAGATTCACTTTTATCACC-3' 5'-CCACTTGATAAGAGGTCCCAAG-3'	Direct Sequencing	60(45)

Figure 1

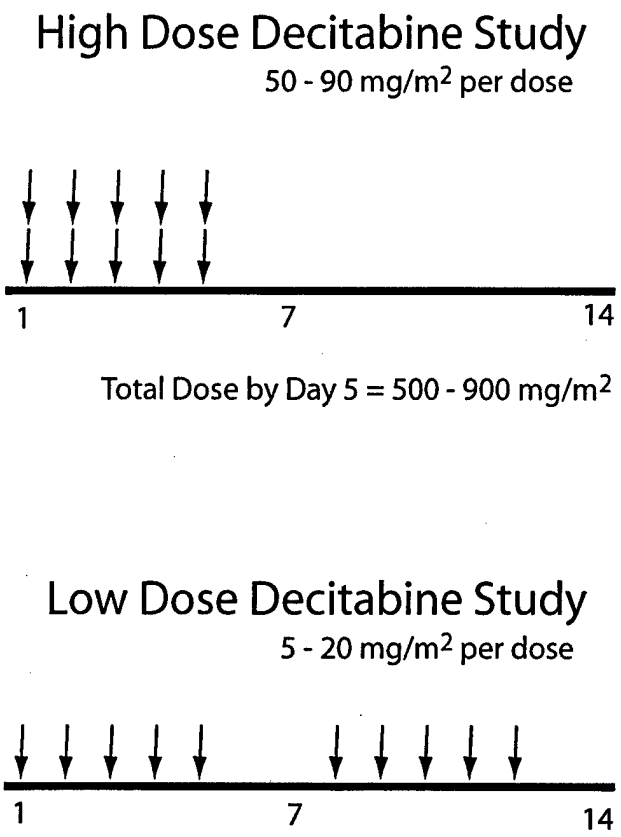

High Dose Decitabine Study
50 - 90 mg/m2 per dose
1
7
14
Total Dose by Day 5 = 500 - 900 mg/m2
Low Dose Decitabine Study
5 - 20 mg/m2 per dose
1
7
14
Total Dose by Day 5 = 25 - 100 mg/m2
Total Dose by Day 12 = 50 - 200 mg/m2

Figure 2A

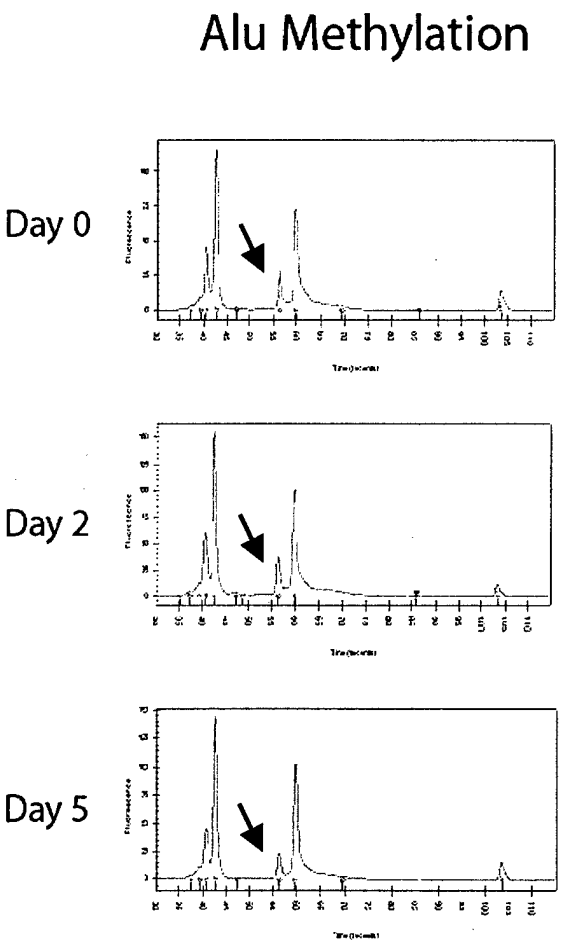

Alu Methylation
Day 0
Day 2
Day 5
COBRA
Capillary Electrophoresis

Figure 2B

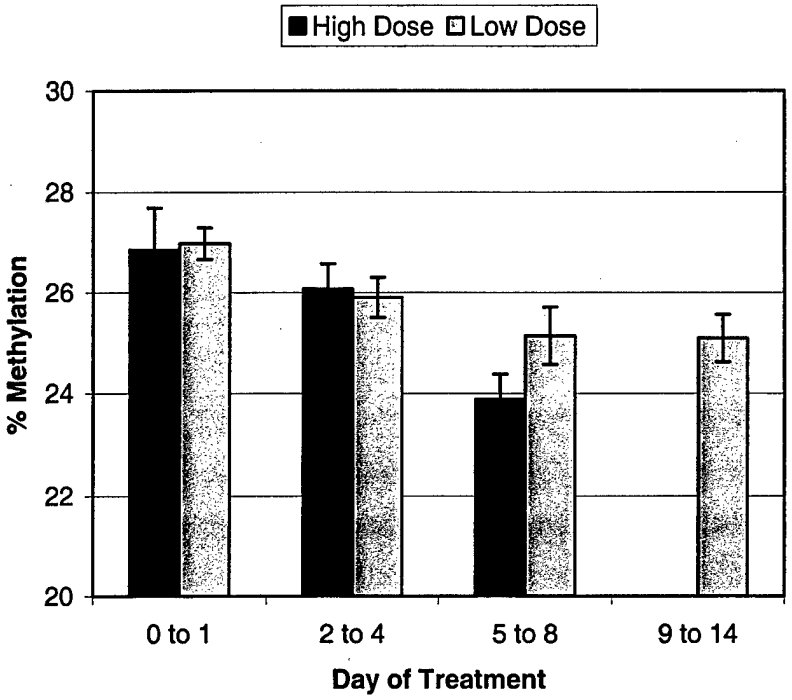

High Dose
Low Dose
30
28
26
24
22
20
% Methylation
0 to 1
2 to 4
5 to 8
9 to 14
Day of Treatment

Figure 3A

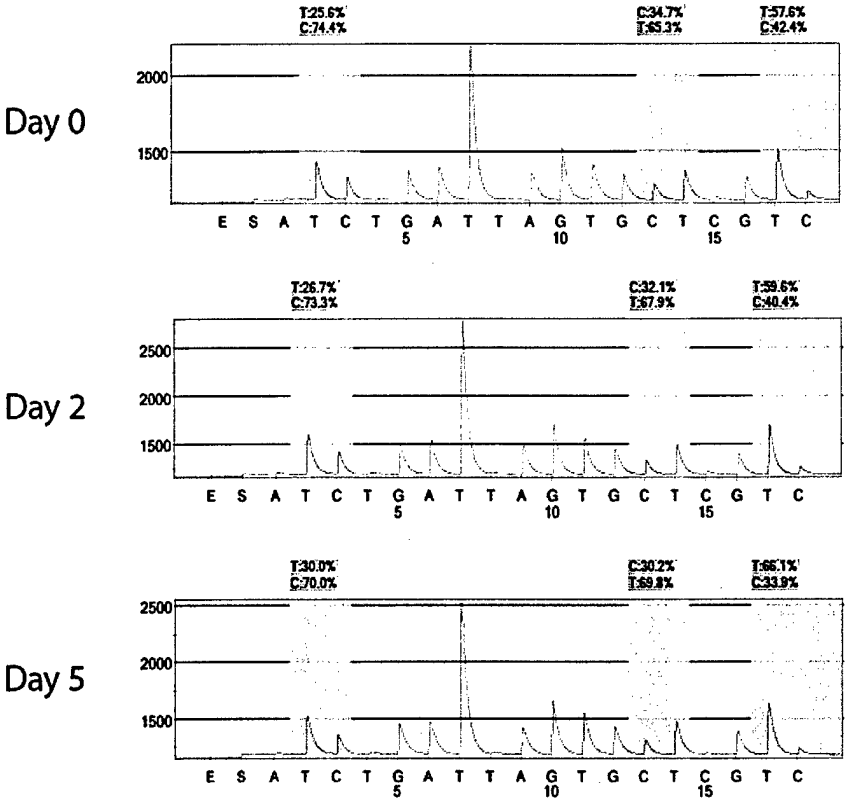

Day 0
T:25.6%
C:74.4%
C:34.7%
T:65.3%
T:57.6%
C:42.4%
2000
1500
E S A T C T G A T T A G T G C T C G T C
5
10
15
Day 2
T:26.7%
C:73.3%
C:32.1%
T:67.9%
T:59.6%
C:40.4%
2500
2000
1500
E S A T C T G A T T A G T G C T C G T C
5
10
15
Day 5
T:30.0%
C:70.0%
C:30.2%
T:69.8%
T:66.1%
C:33.9%
2500
2000
1500
E S A T C T G A T T A G T G C T C G T C
5
10
15

Figure 3B

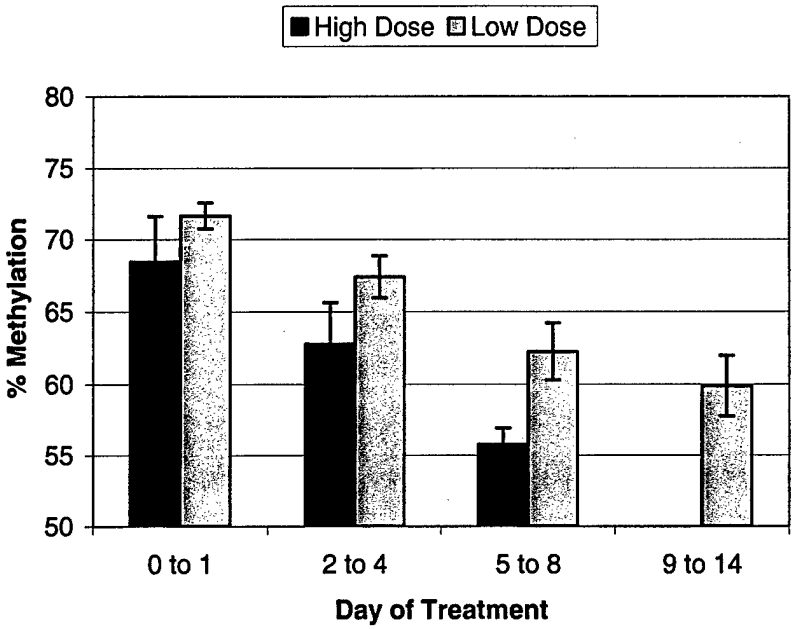

High Dose
Low Dose
80
75
70
65
60
55
50
% Methylation
0 to 1
2 to 4
5 to 8
9 to 14
Day of Treatment

Figure 4

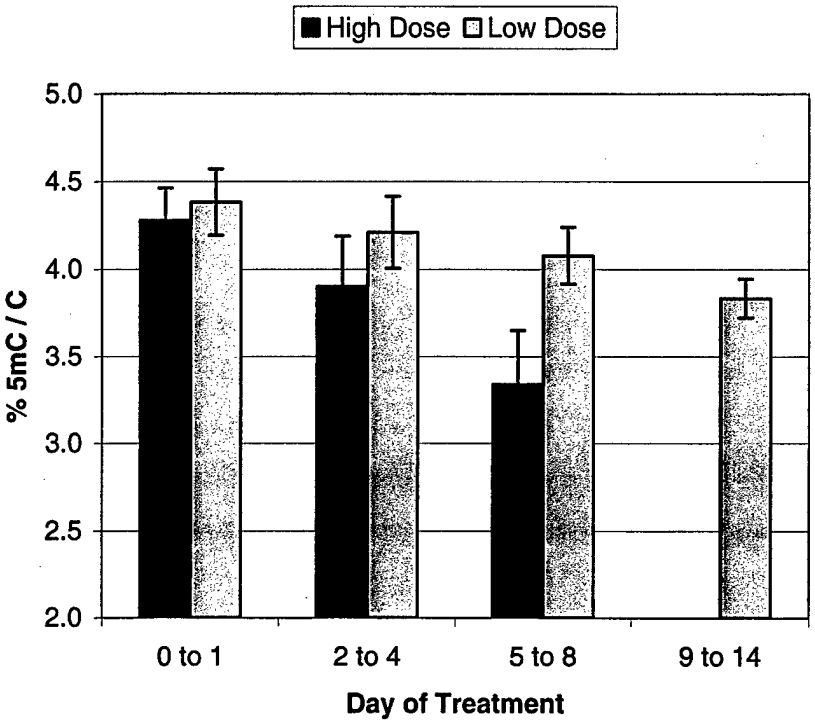

High Dose
Low Dose
5.0
4.5
4.0
3.5
3.0
2.5
2.0
% 5mC / C
0 to 1
2 to 4
5 to 8
9 to 14
Day of Treatment

Figure 5

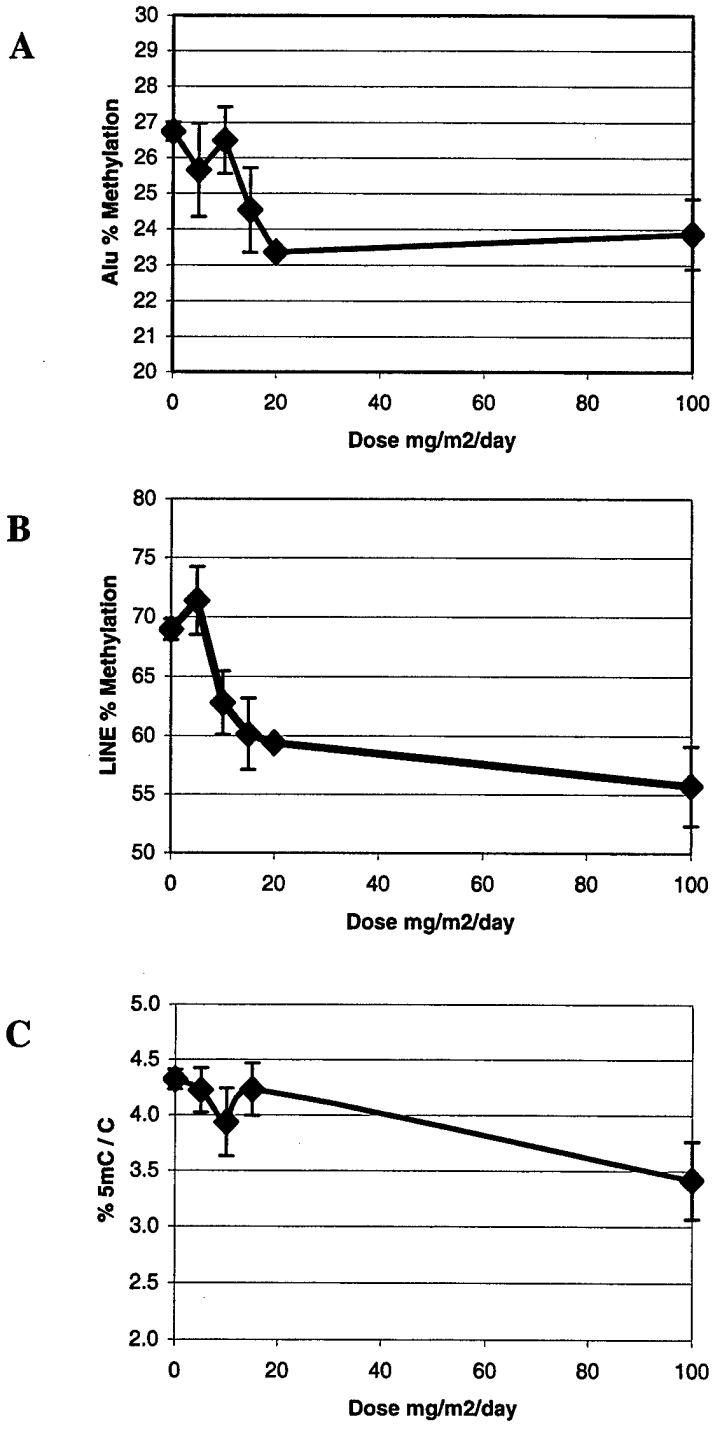

A
Alu % Methylation
30
29
28
27
26
25
24
23
22
21
20
0
20
40
60
80
100
Dose mg/m2/day
B
LINE % Methylation
80
75
70
65
60
55
50
0
20
40
60
80
100
Dose mg/m2/day
C
% 5mC / C
5.0
4.5
4.0
3.5
3.0
2.5
2.0
0
20
40
60
80
100
Dose mg/m2/day

Figure 6ACE

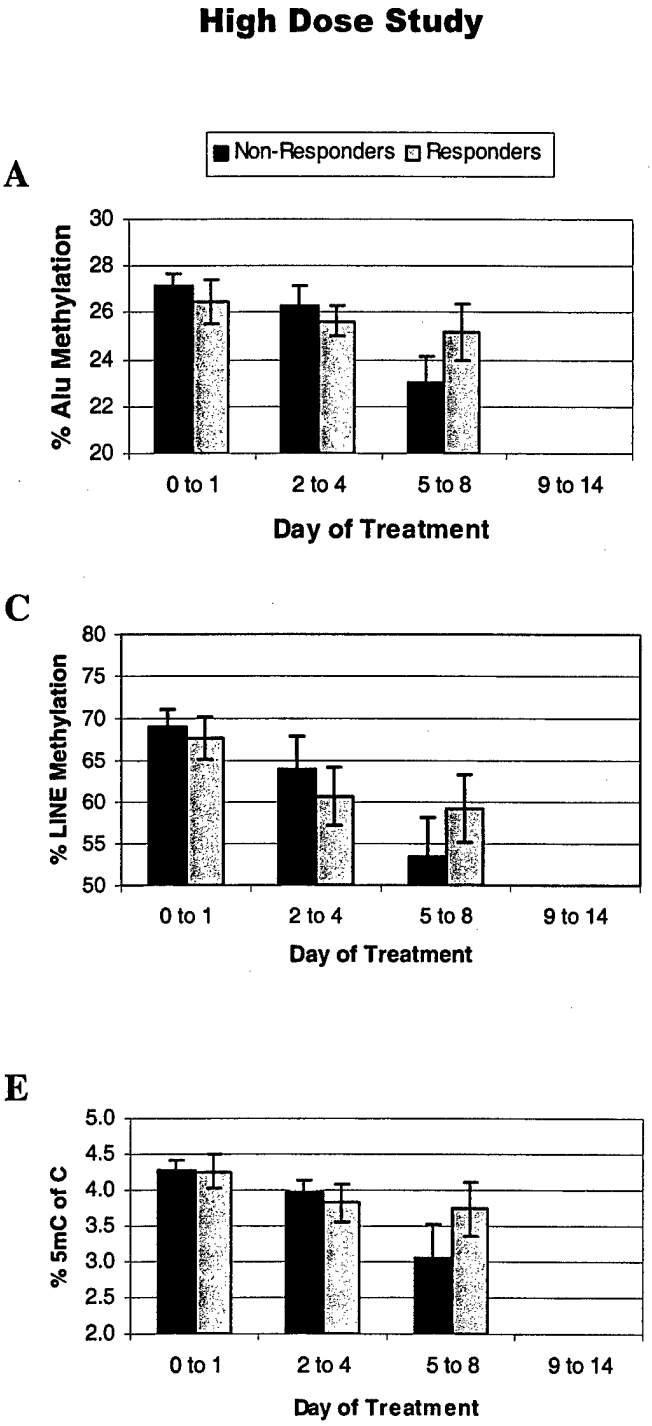

High Dose Study
A
Non-Responders
Responders
% Alu Methylation
30
28
26
24
22
20
0 to 1
2 to 4
5 to 8
9 to 14
Day of Treatment
C
% LINE Methylation
80
75
70
65
60
55
50
0 to 1
2 to 4
5 to 8
9 to 14
Day of Treatment
E
% 5mC of C
5.0
4.5
4.0
3.5
3.0
2.5
2.0
0 to 1
2 to 4
5 to 8
9 to 14
Day of Treatment

Figure 6BDF

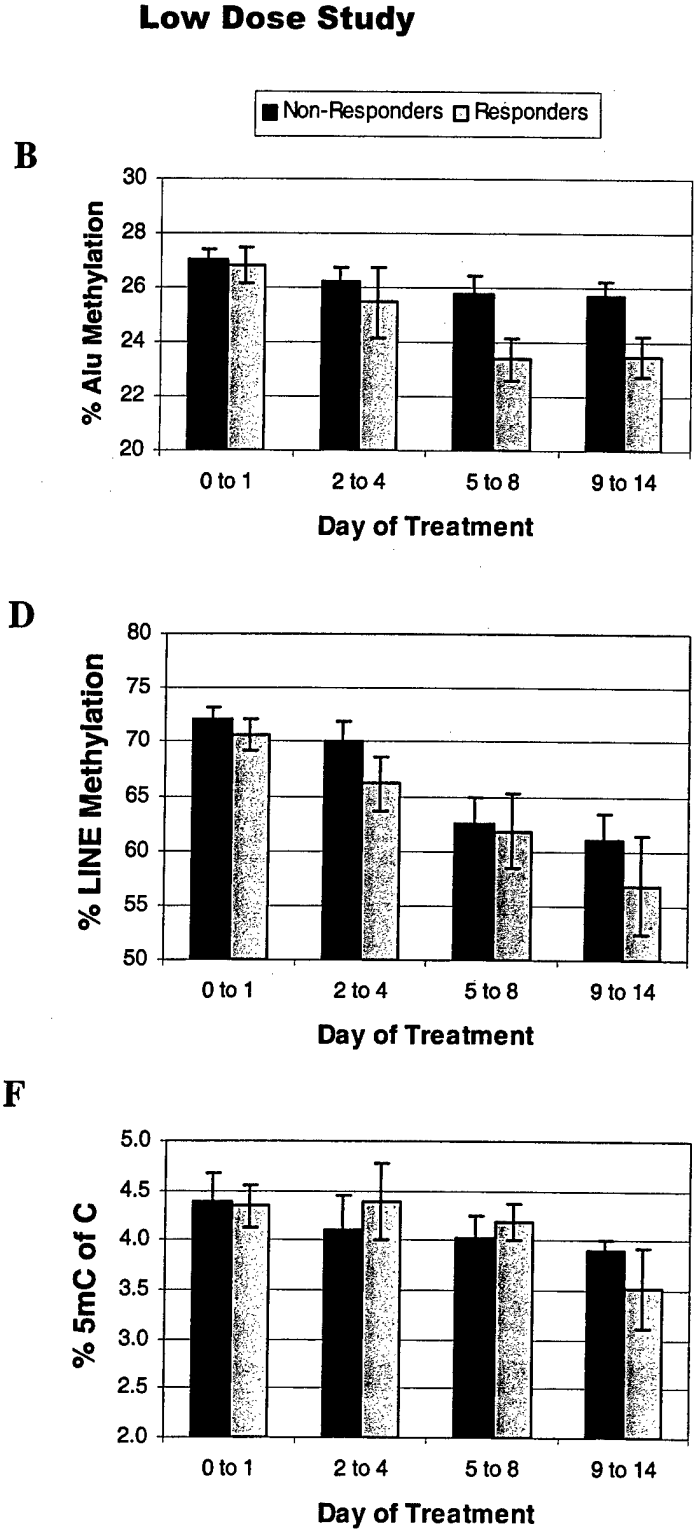

Low Dose Study
Non-Responders
Responders
B
% Alu Methylation
30
28
26
24
22
20
0 to 1
2 to 4
5 to 8
9 to 14
Day of Treatment
D
% LINE Methylation
80
75
70
65
60
55
50
0 to 1
2 to 4
5 to 8
9 to 14
Day of Treatment
F
% 5mC of C
5.0
4.5
4.0
3.5
3.0
2.5
2.0
0 to 1
2 to 4
5 to 8
9 to 14
Day of Treatment

Figure 7AC

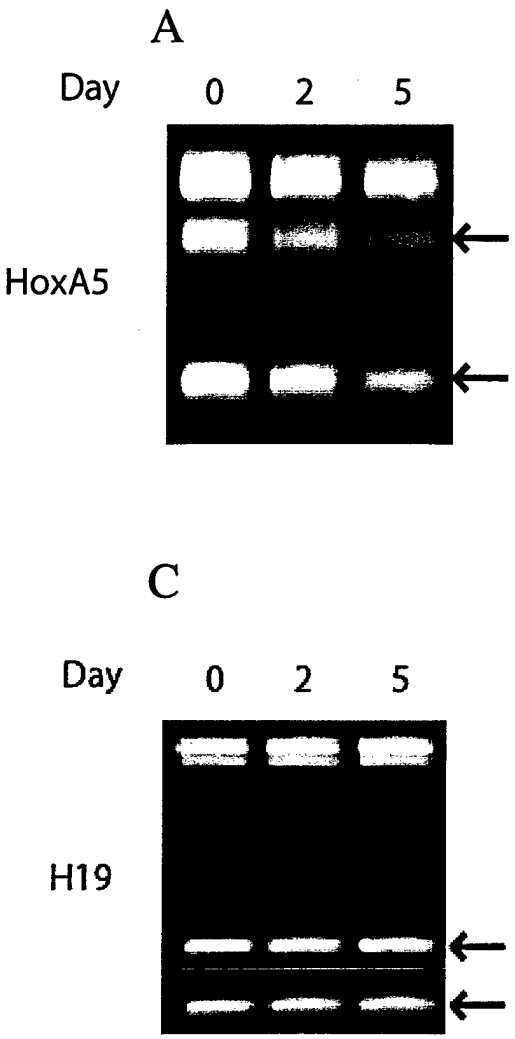


Figure 7B

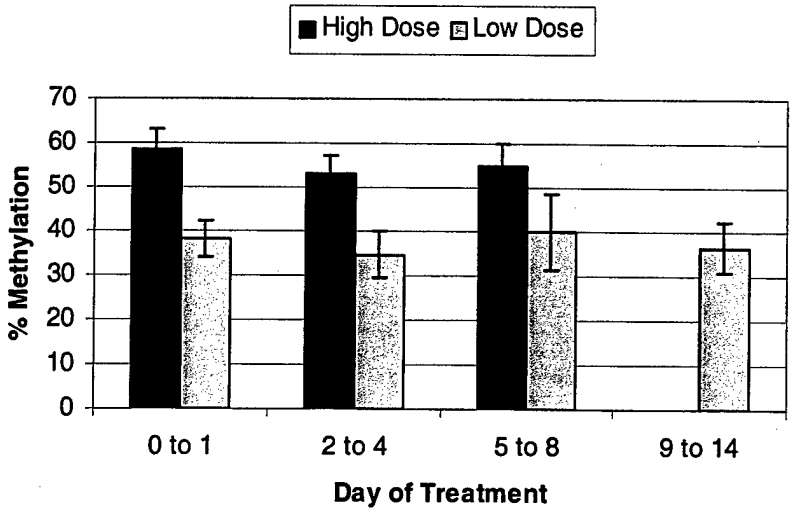

High Dose
Low Dose
70
60
50
40
30
20
10
0
% Methylation
0 to 1
2 to 4
5 to 8
9 to 14
Day of Treatment

Figure 7D

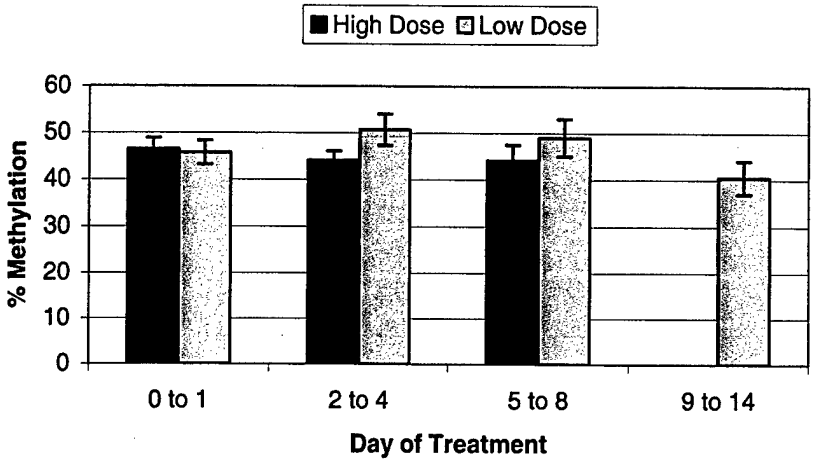

High Dose
Low Dose
% Methylation
60
50
40
30
20
10
0
0 to 1
2 to 4
5 to 8
9 to 14
Day of Treatment

Figure 8A

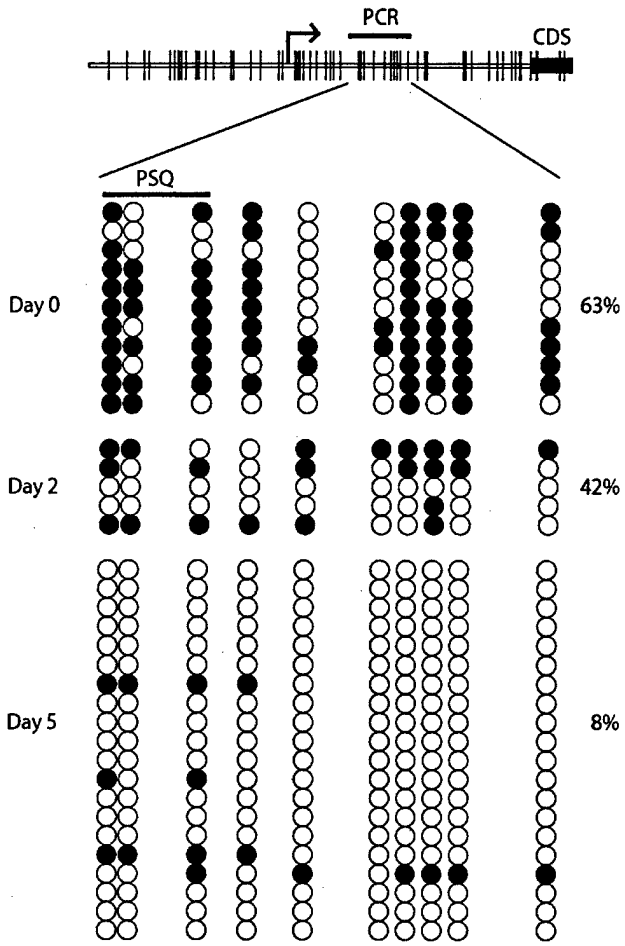
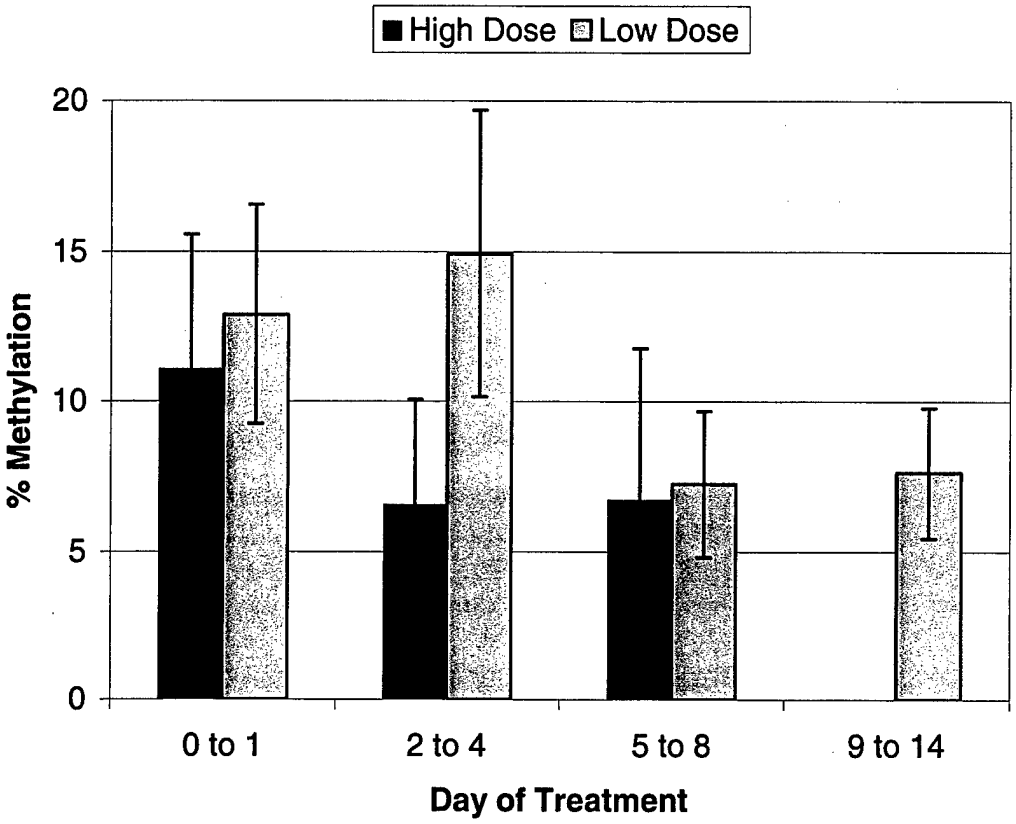


Figure 8B


High Dose
Low Dose
20
15
10
5
0
% Methylation
0 to 1
2 to 4
5 to 8
9 to 14
Day of Treatment

THE UNIVERSITY OF TEXAS
MD ANDERSON
CANCER CENTER

Signature Page

Epigenetic silencing and resistance to imatinib mesylate in CML
LAB04-0035

Core Protocol Data

Study Chair:	Jean-Pierre Issa
Department:	Leukemia
Full Title:	Epigenetic silencing and resistance to imatinib mesylate in CML
Version:	01

THE UNIVERSITY OF TEXAS
M. D. ANDERSON CANCER CENTER
DIVISION OF CANCER MEDICINE

PROTOCOL NAME: Epigenetic silencing and resistance to imatinib mesylate in CML

THE UNIVERSITY OF TEXAS
MD ANDERSON
CANCER CENTER

Protocol Page

Epigenetic silencing and resistance to imatinib mesylate in CML
LAB04-0035

Core Protocol Information

Short Title	Epigenetic silencing and resistance to imatinib mesylate in CML
Study Chair:	Jean-Pierre Issa
Additional Contact:	Laura Sasse
Department:	Leukemia
Phone:	713-745-2260
Unit:	428
Study Co-Chair(s):	Guillermo Garcia-Manero
Full Title:	Epigenetic silencing and resistance to imatinib mesylate in CML
Protocol Type:	Lab Protocol
Protocol Phase:	N/A
Version Status:	Working copy 02/03/2004
Version:	01

Protocol Body

1.0 Objectives

To determine the methylation profile of candidate tumor-suppressor genes in patients with CML treated with Imatinib, and correlate it with response/resistance to this agent.

2.0 Rationale

Markers of drug-resistant disease may provide powerful markers of prognosis in CML. By identifying high/low risk groups, these could direct patients towards alternate forms of treatment, including, potentially, methylation-directed treatments. In this specific aim, we will test the hypothesis that methylation-associated epigenetic silencing of specific genes are prognostic markers in CML patients treated with Imatinib. To test this hypothesis, we will retrospectively study DNA methylation of specific genes in CML patients treated with Imatinib, and correlation methylation with response in CML. Positive results will be confirmed prospectively.

3.0 Location of Sample Collection

Samples will be collected from the Leukemia Tissue Bank at M.D. Anderson.

4.0 Type of Samples to be Collected

Peripheral blood or bone marrow samples at diagnosis have already been collected from patients as part of the Leukemia Tissue Bank. We plan to study promoter methylation of a group of selected genes in 200 patients/year for two years (400 patients) with CML of various phases who have been treated with Imatinib and followed for at least 1 year.

5.0 Number of Samples to be Studied

200 patients/year for two years (400 patients) with CML of various phases who have been treated with Imatinib and followed for at least 1 year

6.0 Eligibility of Subjects

Through phase I/II studies conducted at MDACC over the past 4 years, we have access to over 400 patients with CML in various stages treated with Imatinib (200 in CML-CP, 120 in CML-AP and 80 in CML-BC). This group of patients has a median follow-up in excess of 1 year, and all clinical information is available through a frequently updated database of CML patients seen at MDACC. In addition, we are currently prospectively collecting bone marrow on all new patients with CML seen at MDACC (roughly 100-200/year). Given that all such patients are enrolled in Imatinib trials, we will have access to a completely independent group of 400-500 patients in year 3 of the proposed application. All new patients seen at this institution have bone marrow stored for potential research studies. In addition, a bone marrow biopsy is part of the work-up of all new patients seen here, and we have successfully conducted methylation studies on DNA extracted from paraffin-embedded bone marrow in patients with leukemia. Based on our experience in ALL, we estimate a 'capture' rate of 80% for obtaining diagnostic samples from patients seen at this institution. For the current specific aim, patients will be selected solely based on availability of a bone marrow sample for methylation analysis. The relative ease of modern methods to study DNA methylation allows us to plan to analyze all identified samples for the planned genes. Indeed, we have extensive experience with the analysis of multiple genes in primary cancer samples.

7.0 Data Confidentiality Procedures

All samples will be assigned a unique identifying number that is not the patients' mdeical record number. The samples will remian anonymous during analysis and only the researchers directly involved in the study will have access to the key that correlates the study number with the patient's information.

8.0 Research Plan and Methods

Methylation will be studied by bisulfite conversion followed by both quantitative (COBRA) and sensitive (MSP) methods. Methylation will then be correlated with response and survival in each stage of CML. Genes to be studied include ABL, P15, RIL, TIG1 and a panel of genes currently being cloned from CML cell lines.

9.0 Procedure to Obtain Informed Consent/Waiver of Consent

A waiver of consent is requested because the study is a retrospective laboratory study involoving samples that have already been donated to the Leukemia Tissue Bank. Patients have already given consent to donate these samples for research purposes. In addition, there is minimal risk to the patients because (1) these lab studies do not affect the medical treatment of the patients by the attending physicians, (2) the information collected does not reflect germline events and (3) confidentiality will be preserved through strict adherence to the data confidentiality procedures..

10.0 Related CCSG (NCI Core Grant) Program

11.0 Protocol Manager

Gail Morris, R.N.

Epigenetic silencing and resistance to imatinib mesylate in CML
LAB04-0035

Subtitle:

Request for Waiver of Informed Consent

Protocol Number: **LAB04-0035**
Principal Investigator: **Jean-Pierre Issa**
Protocol Title: **Epigenetic silencing and resistance to imatinib mesylate in CML**

The research involves no more than minimal risk to the subjects.
A waiver of consent is requested because the study is a retrospective laboratory study involoving samples that have already been donated to the Leukemia Tissue Bank. Patients have give consent to donate these samples for research purposes. In addition, there is minimal risk to the patients because these lab studies do not affect the medical treatment of the patients by the attending physicians.

The waiver or alteration will not adversely affect the rights and welfare of the subjects.
The patients have signed informed consents agreeing to donate samples to the Leukemia Tissue Bank for research purposes.

The research could not practicably be carried out without the waiver or alteration.
The majority of patients who have donated samples to the Leukemia Tissue Bank do not live in the immediate locations to M.D. Anderson. Obtaining consent from them would be difficult.

Whenever appropriate, the subjects will be provided with additional pertinent information after participation.
Epigenetic studies are not currently being used for clinical care.

Waiver of Informed Consent Date:	02/26/2004 12:00:00 AM
For IRB Use Only: Waiver Approved: Yes	Reviewer: Walter N. Hittelman

The IRB responsible for review and approval of this waiver was The University of Texas M. D. Anderson IRB #2 IRB00002203

Waiver of Authorization to Use and Disclose Protected Health Information (PHI)

Protocol Number: LAB04-0035
Principal Investigator: Jean-Pierre Issa
Protocol Title: Epigenetic silencing and resistance to imatinib mesylate in CML

1. **The use or disclosure of the PHI involves no more than minimal risk to the individual's privacy. This is based on the following 3 criteria:**

   (a) The research protocol includes adequate plans to protect identifiers from improper use.
   All samples will be assigned a unique identifying number that is not the patients' mdeical record number. The samples will remian anonymous during analysis and only the researchers directly involved in the study will have access to the key that correlates the study number with the patient's information.

   (b) The research protocol includes an adequate plan to destroy the identifiers at the earliest opportunity consistent with conduct of the research.
   The sample ideitifier key will be destroyed when the research samples are exhausted or the research project has been completed.

   (c) The research protocol includes adequate written assurances that the PHI will not be reused or disclosed to any other person or entity, or for other research.
   Protected Health Information will only be used for research purposes by people involved in this research study. The information from this research project will not be shared or dislosed to any other person or entity.

2. **The research could not practicably be conducted without this waiver or alteration.**
   The majority of patients who have donated samples to the Leukemia Tissue Bank do not live in the immediate locations to M.D. Anderson. Obtaining consent from them would be difficult.

3. **The research could not practicably be conducted without access to and use of the PHI.**
   The objective of the laboratory study is to determine the methylation profile of candidate tumor-suppressor genes in patients with CML treated with imatinib medylate, and correlate it with response/resistance to this agent. Access and use of the PHI is critical to the research objective.

Waiver of Authorization Date:	02/26/2004 12:00:00 AM
Walter N. Hittelman	
Print Name of IRB Authorized Individual	Signature
This waiver was reviewed and approved by the following method:	○ Full Committee ● Expedited Review

The IRB responsible for review and approval of this waiver was The University of Texas M. D. Anderson IRB #2 IRB00002203

THE UNIVERSITY OF TEXAS
MD ANDERSON
CANCER CENTER

MEMORANDUM

DATE: June 20, 2000

TO: Guillermo Garcia-Manero, M.D.
Leukemia
Box 61

FROM: Cathy Williams
IRB Coordinator, Office of Protocol Research
Box 38

SUBJECT: Approval of Protocol LAB00-200 Entitled, "Study of DNA Methylation in Acute Lymphoblastic Leukemia"

Official Approval Date: June 19, 2000

Aman U. Buzdar, M.D., Surveillance Committee Chairman, reviewed and administratively approved the above- named and numbered protocol noting that risks to human subjects are minimal and that confidentiality of records will be maintained. If a grant is the basis of your protocol, the grant must be funded before research can begin.

To remain in compliance with federal regulations, each protocol involving human subjects must be reviewed and approved annually by the Surveillance Committee. The Office of Protocol Research will notify you when this protocol needs review. To ensure continuation of your protocol, it is essential that the information requested be provided in advance of the review date.

CAB00-200

HUMAN SUBJECTS CHECKLIST

The purpose of this document is to evaluate information related to the proposed research and determine the extent of review (if any) required by the Surveillance Committee (IRB) in accordance with institutional and federal regulations (45 CFR 46).

Clinical protocols are due in the Office of Protocol Research by 5:00 p.m. on the second or last Monday of the month. After receiving approval from the Clinical Research Committee, the Protocol will be acted upon by IRB.
Documentation of drug or device source approval is the responsibility of the study chairman. Source approval AND Surveillance Committee approval are required before a protocol can be activated.

1 Is this a Diagnostic or Therapeutic Study?	☐ Yes	☒ No
2 **Biosafety check:**		
Does this study include any products manufactured or produced at M. D. Anderson?	☐ Yes	☒ No
Does this protocol use recombinant DNA?	☐ Yes	☒ No
3 **Does this research involve the Use of Educational Tests, Survey Procedures, Interview Procedures or Observation of Public Behavior?**	☐ Yes	☒ No
Are responses recorded in such a manner that the subject can be identified, directly or through identifiers linked to the subject?	☐ Yes	☒ No
Could the subject's responses, if they became know outside the research, reasonably place the subject at risk of criminal or civil liability or be damaging to the subject's financial standing, employability, or reputation?	☐ Yes	☒ No
Does the research deal with sensitive aspects of the subject's own behavior, such as illegal conduct, drug use, sexual behavior, or use of alcohol?	☐ Yes	☒ No
4 Research Involving Human Specimens, Cell Lines or Data	☐ N/A	
a. **Human Specimens**	☒ Yes	☐ No
Will human specimens be obtained specifically for this research?	☐ Yes	☒ No
Will human specimens be obtained from incidental (residual) samples?	☒ Yes	☐ No
Are the data relating to the specimens kept by the investigator in such a way that the patient can be identified?	☐ Yes	☒ No
Does the investigator guarantee to preserve confidentiality?	☒ Yes	☐ No
Total number of patients _____ samples 100 required		
b. **Human Cell Lines** _____ **Data** X	☒ Yes	☐ No
Are cell lines or data obtained from a public repository or source?	☐ Yes	☒ No
Can the cell lines or data be linked directly to the subject?	☐ Yes	☒ No
5 **Could the research lead to a patentable procedure or product?** (If yes, a copy of the proposal should be submitted to the Office of Technology Department, Box 510.)	☐ Yes	☒ No

The information provided regarding this research activity is true and correct to the best of my knowledge.

Title of Proposal: Study of DNA Methylation in Acute Lymphoblastic Leukemia

Principal Investigator: Garcia-Manero, M.D. 5-31-00 (Name/Signature/Date) Box 61 Extension 53424 ####

Collaborators: Issa, M.D.

Department Head Approval: Kantarjian, M.D. 5-31-00 (Name/Signature/Date)

Surveillance Committee (IRB) 6-6-00 Exemption 2 ____ Exemption 4 ____ Other Lab.

AMAN U. BUZDAR, M.D.

CAB00-200

Guillermo Garcia-Manero, M.D.

1-Background.

CpG methylation is the addition of a methyl group to the fifth position of the cytosine ring in a cytosine guanine dinucleotide (CpG). CpG dinucleotides (CpGs) have a peculiar distribution in the human genome. Overall, CpG sites are present at a lower frequency than expected in vertebrate DNA [1]. CpG sites are found in two distinct areas: Most CpG sites are located in peri-centromeric satellite DNA and within repeat elements (Alus and LINEs) present in non-coding intergenic areas. Most of these are methylated in normal tissues. This methylation appears to play a role in chromosomal stability, and has also been proposed to serve as a defense mechanism against the recombinational properties of these repeat elements [2]. CpG sites can also be found in close proximity to the 5'end of many genes, involving both promoter regions and 5' untranslated regions. In these areas, a near normal representation of CpGs can be found, and for that reason they are known as CpG islands. It is estimated that there are close to 45,000 of these islands in the human genome [3]. Most of these CpG islands are not methylated in normal tissues, regardless of the expression status of the associated gene [1].
The association between DNA methylation and epigenetic gene silencing has raised considerable recent interest in this phenomenon. Epigenetic silencing refers to stable, non-genetic inactivation of gene expression that is clonally inherited and thought to be irreversible under most normal situations [4]. A role for DNA methylation in silencing was revealed by the observed inverse relationship between the transcriptional activity of a particular promoter and its methylation status [5]. CpG island methylation related silencing is important for imprinting [6] and X-chromosome inactivation in women [7]. Besides this physiological role, aberrant CpG island methylation has been implicated in certain disease processes, such as the Fragile X syndrome [8], aging [9] and in cancer [10], [11] . DNA methylation has been implicated in oncogenesis by silencing the expression of genes crucial for cell function, such as tumor suppressor genes. This phenomenon represents a molecular alternative to gene mutation, and/or deletion for the inactivation of critical genes.
The regulation of CpG island methylation is relatively poorly understood, and little is known about the mechanisms that lead to aberrant methylation in cancer. Several enzymes have been implicated in the methylation of CpGs. They are known as DNA methyltransferases. *DNMT1* was the first enzyme cloned with the capacity to add methyl groups to cytosines [12]. It is now thought that *DNMT1* is primarily a maintenance enzyme, responsible for reproducing patterns of DNA methylation after replication. It does so by using hemimethylated DNA as a template after DNA replication, and it may have little impact on the generation of de novo methylation patterns. Recently, two novel DNA methyltransferases have been cloned, *DNMT3A* and *DNMT3B* [13]. These two enzymes have been shown to have de novo methylation activity, and their lack of expression is lethal in a knock out mouse model [14]. Of interest, the ICF syndrome, a rare autosomal recessive disorder that consists of immunodeficiency, centromeric chromosomal instability and facial abnormalities has recently been shown to be caused by loss of function mutations in *DNMT3B* [14], [15]. Hypomethylation, primarily of centromeric DNA, is a central characteristic of this disease and likely plays a role in the generation of the phenotype [16]. The expression of *DNMT3A* and *DNMT3B* does not correlate with CpG island methylation in cancer [17], but it is not known whether activating mutations of these genes are present in human neoplasms.

The methylation analysis of multiple genes in individual patient samples has revealed considerable variation in the degree of methylation, suggesting that the process is not random, but likely results from specific defects in methylation control. Thus, an extensive analysis of colon cancer has revealed a subset of cases that evolves with a high degree of methylation and silencing – a phenotype termed CpG Island Methylator Phenotype [18]. This concept has now been verified in other tumor types, including gastric [19] and hepatocellular cancer, as well as hematopoietic malignancies. The importance of this phenomenon is that the clinical and biological characteristics of the different methylation groups appear to be substantially different [20], and this may have clinical implications such as different prognosis, and different responses to specific therapeutic interventions. In acute leukemias, in particular, it is clear that several genes, including ER; p15 and p16 are abnormally methylated in an important subset of patients [21].

2-Overall objectives.

1)-To determine the methylation status of multiple genes from a cohort of patients with ALL treated homogeneously at MD Anderson Cancer Center. All the patients characteristics and response to the treatment are known, and therefore multivariate analysis linking the methylator characteristics of the patients with their other biological and clinical characteristics will be performed. 2)-To sequence DNMT3A and 3B from patients with ALL and other hematological malignancies.

3-Specific aims.

The main two hypotheses of the proposed study are:

1) That patients with acute lymphoblastic leukemia (ALL) can be classified according to their methylator phenotype, and that this phenotype has clinical implications (i.e. shorter/longer disease free survival, overall survival or better response to treatment).

2) That the aberrant methylator phenotype is mediated by genetic changes (mutation/deletion) in two recently cloned de-novo DNA methyltransferases.

Based on these two main hypotheses, our specific aims are the following:

1) To measure promoter-associated CpG island methylation of several genes in patients with ALL uniformly treated with the hyperCVAD program (UTMDACC protocol DM93-077). The study will include the methylation analysis of at least the following genes known to be methylated or down regulated in ALL: estrogen receptor (ER), p15, p16, p73, MDR-1, thrombospondin (THBSI) and c-abl.
2) To correlate the above findings with the clinical and biological characteristics of the patients, as recorded for patients enrolled in protocol DM 93-077
3) To sequence the cDNA of DNMT3A and DNMT3B from patients with ALL and particular aberrant methylator phenotypes.

4-Rationale and Preliminary Results.

ALL is a hematopoietic malignant disorder. The prognosis of children with this disease has improved dramatically thanks to the use of intensive systemic chemotherapy [22]. Despite these advances, the outcome of adult patients with this disease still is relatively poor, probably due the higher prevalence of poor prognostic factors, such as the presence of the Philadelphia chromosome. One of the factors that contributes to the difficulty in evaluating and treating adult patients with ALL is the heterogeneity of this disease, represented by the several immunophenotypes [23] in which ALL can be divided. It is well established that each different phenotype has a different prognosis and requires different therapy [24]. Therefore a molecular classification of ALL using both genetic and epigenetic changes that could be correlated with a particular outcome as well as with the need for specific therapy (such as hypomethylating agents or histone deacetylase agents) will be of great benefit when evaluating these patients.

Although several studies have evaluated the methylation status of individual genes in ALL, such as p15 [25], no study has comprehensively evaluated multiple genes per patient and therefore has explored the concept of the methylator phenotype in this disease. Our study also benefits from the fact that the samples used in this study correspond to patients treated with our hyperCVAD program [24]. These allow access to samples from a group of patients that have been treated homogeneously and that have been thoroughly evaluated and followed. As we have access to all the patients characteristics, we will be able to establish correlations between the different methylator characteristics and their clinical-biological characteristics.

Preliminary studies.

Using bisulfite treated DNA and specific primers (see below), we have analyzed the methylation status of the following genes: ER, p15, p16, MDR1, THBSI, p73 and c-abl. We have analyzed 49 patients treated with the hyperCVAD program (protocol DM 93-077) [24]. 9 patients (18.4%) have no methylation in any of the 7 genes analyzed, 14 patients (28.6%) have only one gene methylated, 10 patients (20.4%) have 2 genes methylated, 8 patients (16.3%) have 3 genes methylated and 8 patients (16.3%) have 4 or more genes methylated. The results of the number of patients with methylation of a specific gene are shown below in Table 1.

TABLE1. NUMBER OF PATIENTS AND % METHYLATED AT A PARTICULAR GENE

ER	p15	p16	MDR	TBP	P73	c-abl
22 (44.9%)	10 (20.4%)	2 (4.1%)	25 (51%)	16 (32.7%)	13 (26.5%)	4 (8.2%)

Also available for these patients are immunophenotypes, cytogenetics, disease free survival (DFS), overall survival (OS) and response to treatment. This information will allow us to perform in depth correlations between specific methylation patterns and patient characteristics. At the time of the writing of this proposal, we have calculated the DFS and OS for patients with methylation of a particular gene versus unmethylation, as well as depending on the number of genes methylated.

Despite the small sample size analyzed, several important trends have already been detected. The most significant findings obtained so far are: 1) patients with no methylation of p73 have a statistically significant better OS (54% vs. 28% at 351 weeks, p=0.008), compared with those with methylation of p73; 2) there is a statistically significant difference (p= 0.042) for patients with methylation of THBSI, in terms of better OS (62% vs. 38% at 351 weeks) and 3) although there are no differences in terms OS for patients with MDR methylation compared with those without methylation, there is a strong trend (p= 0.078 by the Logrank test) for a longer DFS for patients with MDR methylation. Other genes showed no impact on survival or only a marginally significant trend.

In view of the significant results findings for p73 and THBSI, we have grouped the patients into 4 categories based on their methylation status: 1) patients with methylation of THBSI but no methylation of p73; 2) patients with methylation of p73 without methylation of THBSI; 3) patients with methylation of both genes and 4) patients with methylation of neither. The results of this grouping, although preliminary, are remarkable. Patients with only methylation of the thrombospondin gene have a strong trend towards better OS (76% at 390 weeks, p=0.08) as compared with the other 3 possibilities and a significantly better DFS (64% at 324 weeks, p=0.002). See graphs 1 and 2 on respective appendixes for graphic display. These results are remarkable, when the long-term survival for adult patients with ALL is around 35%. By contrast, patients with p73 methylation had a strikingly poor outcome (80% dead during the first 78 weeks), while patients with methylation of neither gene have an intermediate OS and DFS. These preliminary results, which need to be validated using larger number of patients and analyzed by multivariate analysis, nevertheless suggest that methylation profiling will provide important clinical information in ALL.

We have begun sequencing the cDNA of DNMT3A and DNMT3B. These two enzymes have the capacity of de novo methylating DNA. Both of them have recently been cloned, sequenced [13] and results of the knockout models reported [14]. These data show that DNMT3A is located in chromosome 2p23 and DNMT3B in 20q11.2. Both are frequent areas of chromosomal abnormalities in adult leukemias. Of note, mutations in DNMT3B have been associated with the development of a rare hereditary syndrome known as ICF (see above,[14]). So far we have sequenced around 600 bps of the c-terminus end of both genes without finding any abnormalities. The cDNA that we are using now is derived from cell line extracts to optimize our reactions. The sequencing of the PCR generated fragments has been performed at the Core Facility at UTMDACC with excellent results. Our plan is to completely sequence the cDNA of both enzymes from a group of patients with ALL with known methylator data.

5 & 6-Detailed outline of specific procedures and Methodology.

The techniques to detect and quantify DNA methylation are described in the following website: www.mdanderson.org\leukemia\methylation. This website is kept by Dr. Jean-Pierre Issa and is used worldwide as one of the key reference sites for DNA methylation. In summary, DNA is treated with bisulfite, which results in the conversion of unmethylated cytosines to uracil, leaving most methylated cytosines intact [26]. Using specific PCR primers followed by DNA digestion by restriction enzymes, we can perform quantitative [27] and very sensitive [28] detection of gene specific methylation. The overall protocol is as follows: 1) DNA extraction using standard phenol-chloroform methods; 2) bisulfite treatment of the DNA (this is performed over 16 hours); 3) PCR using specific primers designed based on the sequence of the gene specific CpG island and restriction site map (also available at www.mdanderson.org\leukemia\methylation) ; 4) Gel separation using polyacrilamide gels and 5) Image analysis using digital densitometric analysis. Except for p73, where we have to use a non-quantitative technique, we can quantify the amount of methylation present in the CpG island being amplified. Samples are obtained from two sources: residual DNA from samples obtained for clinical molecular analysis, and frozen cells in a tissue bank kept by Dr. Kornblau from the Department of Bone Marrow Transplantation at UTMDACC. Methylation results are correlated with the patients characteristics and DFS and OS as described above. Statistical analysis will be performed using regression analysis, and multivariate analysis. We believe that our PCR technology is the most advanced, rapid and sensitive approach to evaluate CpG island methylation. This technology has several advantages over Southern blot, the classic technique to study DNA methylation. First it requires small quantities of DNA, second, there is no need for radioactive material and third it allows the study of multiple genes from a single sample. The main difficulties and limitations of the study are the retrospective nature of the study, and the fact that not all patients on study will be able to be studied because of sample availability. Obviously we cannot correct those two problems. If after evaluating 80 to 100 samples, we still need to expand our study, our techniques can be performed with good results from DNA extracted from paraffin blocks as has been done in Dr. Issa's laboratory. This will give us access to samples from all the patients treated on the hyperCVAD protocol, through analysis of paraffin embedded bone marrow biopsies.

7-Relevance to Leukemia.

The importance of this study is based on its long-term applications. The first specific aim is to establish the clinical-biological value of the methylator phenotypes in ALL. If the preliminary data (see above) is corroborated by the proposed study this information could be used to develop several new long-term projects. They will include: 1) the development of a new molecular classification of ALL, which will allow targeting the poor risk patients with alternate forms of therapy up front; 2) the development of targeted therapies using hypomethylating agents for patients with aberrant methylator phenotypes at high risk for disease progression and 3) the design of molecular techniques, using plasma or serum, to evaluate disease progression, minimal residual disease and response to therapy in these patients.

From a more basic perspective, the knowledge that methylation of a particular gene (as in our case the p73 gene, see below) may confer the patient a worse prognosis, will lead to investigate the biological reason for that phenomenon, and potential ways to overcome it. Lastly, the sequencing of DNMT3A and DNMT3B may lead to the discovery of activating mutations that may provide an

enzymatic basis for the aberrant methylator phenotype. Such knowledge will have profound implications in the field of DNA methylation as well as in cancer.

8-Collaboration.

We have established a formal collaboration with Dr. Terri Smith, Department of Statistics at MD Anderson Cancer Center. Dr Smith will oversee the statistical analysis, include the multivariate analysis in this study.

9-References.

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THE UNIVERSITY OF TEXAS
MD ANDERSON
CANCER CENTER

MEMORANDUM

DATE: July 31, 2001

TO: Jeanne Rothberg/Guillermo Garcia-Manero, M.D.
Department of Leukemia
Box 428

FROM: Criselda Owens CO
IRB Coordinator, Office of Protocol Research
Box 38

SUBJECT: Approval of Protocol LAB01-423 Entitled, "Use of Stored Samples from Patients with Leukemia and Myelodysplastic Syndromes to Study DNA-Methylation"

Official Approval Date: July 27, 2001

Richard J. Ford, M.D., Surveillance Committee Vice Chairman, reviewed and administratively approved the above- named and numbered protocol noting that risks to human subjects are minimal and that confidentiality of records will be maintained. If a grant is the basis of your protocol, the grant must be funded before research can begin.

To remain in compliance with federal regulations, each protocol involving human subjects must be reviewed and approved annually by the Surveillance Committee. The Office of Protocol Research will notify you when this protocol needs review. To ensure continuation of your protocol, it is essential that the information requested be provided in advance of the review date.

Please note that a waiver of informed consent has been granted

THE UNIVERSITY OF TEXAS
MD ANDERSON
CANCER CENTER

MEMORANDUM

TO: Criselda Molina
IRB Coordinator
Box 38

FROM: Laura Sasse
Sr. Secretary to Dr. Jean-Pierre Issa
Box 428

DATE: July 11, 2001

RE: Laboratory Protocol titled, "Use Of Stored Samples From Patients With Leukemia And Myelodysplastic Syndromes To Study DNA-Methylation."

I am submitting the above mentioned laboratory protocol. I hope this protocol can be approved expeditiously. If you need to contact me, my phone number is 5-2460. Thank you.

THE UNIVERSITY OF TEXAS
M.D. ANDERSON CANCER CENTER

DIVISION OF MEDICINE

USE OF STORED SAMPLES FROM PATIENTS WITH LEUKEMIA AND MYELODYSPLASTIC SYNDROMES TO STUDY DNA-METHYLATION.

1-OBJECTIVES
2-BACKGROUND
3-METHODS
4-REFERENCES

PRINCIPAL INVESTIGATOR:

Guillermo Garcia-Manero, M.D.

CO-PRINCIPAL INVESTIGATOR:

Jean-Pierre Issa, M.D.

1-Objectives.

-To study the DNA-methylation characteristics of multiple genes from patients with leukemia and myelodysplastic syndromes.

-To obtain the DNA, RNA and protein needed to perform these studies from samples stored in cell/tissue banks.

-To clone new genes abnormally methylated in cancer.

2-Background

We are actively investigating the methylator characteristics of patients with acute leukemias and myelodysplastic syndromes. We have found, in both acute lymphocytic leukemia and acute myelogenous leukemia, important correlates between particular methylator phenotypes and clinico biological characteristics in patients with these diseases. Results from these studies may lead to a new molecular classification of these disorders, the generation of new techniques to detect minimal residual disease and the development of new treatments using hypomethylating agents. These investigations may allow the cloning of abnormally methylated genes in leukemia and cancer in general.

CpG methylation is the addition of a methyl group to the fifth position of the cytosine ring in a cytosine guanine dinucleotide (CpG). CpG sites are found in close proximity to the 5′end of many genes, involving both promoter regions and 5′ untranslated regions. The field of DNA methylation has generated considerable interest recently thanks to several conceptual and technical advances. First, the discovery that epigenetic gene silencing through DNA methylation plays a role in the development of several disease entities, including cancer, has triggered clinical interest in this phenomenon. Second, the development of new technologies to analyze the methylation state of multiple genes in a relatively short time, and to detect DNA methylation abnormalities in serum, has raised the hope of rapidly finding clinical applications for this research. Third, the cloning of several DNA methyltransferases, enzymes with the capacity of methylating DNA, and the discovery of mutations in one of these in a rare hereditary syndrome has increased interest in the role of the methylation machinery in disease. Finally, the possibility of reversing DNA methylation changes pharmacologically has opened new areas of research in the treatment and prevention of cancer.

The association between DNA methylation and epigenetic gene silencing has raised considerable recent interest in this phenomenon. Epigenetic silencing refers to stable, non-genetic inactivation of gene expression that is clonally inherited and thought to be irreversible under most normal situations (1). A role for DNA methylation in silencing was revealed by the observed inverse relationship between the transcriptional activity of a particular promoter and its methylation status (2). CpG island methylation related silencing is important for imprinting (3) and X-chromosome inactivation in women (4). Besides this physiological role, aberrant CpG island methylation has been implicated in certain disease processes, such as the Fragile X syndrome (5), aging (6) and in cancer

(7,8). DNA methylation has been implicated in oncogenesis by silencing the expression of genes crucial for cell function, such as tumor suppressor genes. This phenomenon represents a molecular alternative gene mutation, and/or deletion for the inactivation of critical genes. DNA methylation appears to induce silencing primarily by attracting a protein complex that includes methylated DNA binding proteins and histone deacetylases, ultimately resulting in the formation of a closed chromatin structure (9).

From the above information, it is clear that the analysis of the methylation state of specific genes in diverse disease entities can potentially provide useful clinical information, similar to the analysis of gene mutations or other abnormalities in these same disorders. For example, methylation of specific genes may provide prognostic information (10), and could potentially be used to screen for the presence of cancer. Therefore, the development of rapid and sensitive techniques to study DNA methylation is important for such translational research. Over the past few years, the study of DNA methylation has been greatly facilitated by the realization that bisulfite treatment of DNA results in the conversion of unmethylated cytosines to uracil, leaving most methylated cytosines intact (11). Several bisulfite-PCR based techniques have now been developed, that allow quantitative (12) or very sensitive (13) detection of gene specific methylation. These developments are providing a more complete picture of DNA methylation changes in cancer and their potential clinical implications.

The methylation analysis of multiple genes in individual patient samples has revealed considerable variation in the degree of methylation, suggesting that the process is not random, but likely results from specific defects in methylation control. Thus, an extensive analysis of colon cancer has revealed a subset of cases that evolves with a high degree of methylation and silencing – a phenotype termed CpG Island Methylator Phenotype (CIMP) (14). This concept has now been verified in other tumor types, including gastric (15) and hepatocellular cancer, as well as hematopoietic malignancies. The importance of this phenomenon is that the clinical and biological characteristics of the different methylation groups appear to be substantially different (16), and this may have clinical implications such as different prognosis, and different responses to specific therapeutic interventions.

3. Methods

3a- Patient confidentiality.

Samples will correspond to patients with known diagnoses of leukemia or myelodysplastic syndrome identified from the Department of Leukemia Patient Database. Patient medical number will changed to a code that could only be decipher by the principal investigators, **thus preserving patient confidentiality.**

It is important to note that most of the patients to be evaluated in these studies have succumbed to their diseases, and therefore obtaining consent for sample use is not possible.

3b--DNA, RNA and protein will be extracted using standard laboratory protocols.

-As most of out studies include DNA analysis; we detailed our ongoing laboratory protocol for that DNA extraction:

After incubation, an equal volume of chloroform-isoamyl alcohol (24:1) will be added.
-Mix well and separate phases by centrifugation, the upper phase will be collected.
-Then 2 volumes of cold absolute ethanol and 1/10 vol. of 3 M sodium acetate will be added. DNA will be precipitated at –20C overnight.
-DNA is pelleted by centrifugation, dried and resuspended in water.

3c-Bisulfite treatment of DNA.

-Our techniques to study DNA-methylation are described in the website: **www.mdanderson.org\leukemia\methylation**. In summary, DNA is treated with bisulfite, this results in the conversion of unmethylated cytosines to uracil, leaving most methylated cytosines intact. Using specific PCR primers followed by DNA digestion by restriction enzymes, we can perform quantitative and very sensitive detection of gene specific methylation. The overall protocol is as follows: 1) bisulfite treatment of the DNA (this is performed over 16 hours); 3) PCR using specific primers designed based on the sequence of the gene specific CpG island and restriction site map; 4) Gel separation using polyacrilamide gels and 5) Image analysis using digital densitometric analysis. We can quantify the amount of methylation present in the CpG island being amplified

4. References

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ORA

01.15
Waiver of Informed Consent

Section: 01.0 - Regulatory Affairs/Human Subject Research

Request for Waiver of Informed Consent

Protocol # (entered by OPR)

Principal Investigator: Guillermo Garcia-Manero, M.D.
Protocol Name: Use of Stored Samples From Patients With Leukemia And Myelodysplastic Syndromes To Study DNA Methylation.
Please provide information substantiating the following. Use additional pages if needed.
The research involves no more than minimal risk to the subjects. Samples already stored unfortunately most patients have succumbed to their disease and therefore re-consenting is not possible.
The waiver or alteration will not adversely affect the rights and welfare of the subjects.
The research could not practicably be carried out without the waiver or alteration; and
Whenever appropriate, the subjects will be provided with additional pertinent information after participation.
For IRB use only: **Waiver Approved** (Yes) **No** **Reviewer:** AMAN U. BUZDAR, M.D 8·2·01

THE UNIVERSITY OF TEXAS
MD ANDERSON
CANCER CENTER

MEMORANDUM

DATE: September 8, 2000

TO: Guillermo Garcia-Manero, M.D.
Department of Leukemia
Box 61

FROM: Criselda Molina CM
IRB Coordinator, Office of Protocol Research
Box 38

SUBJECT: Approval of Protocol LAB00-271 Entitled, "Use of Paraffin-Embedded ARchival Samples From patients with Leukemia and Myelodysplastic Syndromes to Study DNA-Methylation"

Official Approval Date: September 6, 2000

The Surveillance Committee reviewed and administratively approved the above- named and numbered protocol noting that risks to human subjects are minimal and that confidentiality of records will be maintained. If a grant is the basis of your protocol, the grant must be funded before research can begin.

To remain in compliance with federal regulations, each protocol involving human subjects must be reviewed and approved annually by the Surveillance Committee. The Office of Protocol Research will notify you when this protocol needs review. To ensure continuation of your protocol, it is essential that the information requested be provided in advance of the review date.

TO: AMAN BUZDAR, M.D.
CHAIRMAN IRB.

FROM: GUILLERMO GARCIA-MANERO, M.D.
DEPARTMENT OF LEUKEMIA, BOX 61

As a follow up of our conversation regarding protocol **"Use of paraffin-embedded archival samples from patients with leukemia and myelodisplastic syndromes to study DNA-methylation"**, it will be next to impossible to obtain signed consent for the use of this archival material as the vast majority of the patients have succumbed to their diseases and for those that are alive it will be extremely difficult to contact them to obtain consent. Therefore, I ask you to grant approval to the above mentioned protocol.

Yours sincerely,

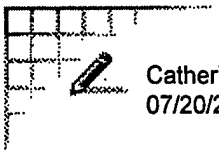
8-29-00

Guillermo Garcia-Manero, M.D.

Add to IRB agenda tomorrow for my Presentation to IRB.

9.5.00

AMAN U. BUZDAR, M.D.



Catherine M. Scherer
07/20/2000 04:25 PM

To: Guillermo Garcia-Manero/MDACC@MDACC
cc: Criselda Molina/MDACC@MDACC

Subject: New Lab Study Entitled, "Use of Paraffin-Embedded Archival Samples From Patients With Leukemia and Myelodysplastic Syndromes to Study DNA-Methylation"

Dr. Aman Buzdar, IRB Chair, reviewed your new lab study and determined that a tissue consent must be included with the protocol. This form (Consent Form for Use of Tissue for Future Research) can be found in the OPR Manual in Lotus Notes. Please submit this consent to me at Box 38. Be sure to type the title of the study in the box in the upper right corner of the consent.

Your assistance is appreciated.

Cathy Williams
IRB Coordinator
Office of Protocol Research (Box 38)
x4-4607

LAB00-271

HUMAN SUBJECTS CHECKLIST

The purpose of this document is to evaluate information related to the proposed research and determine the extent of review (if any) required by the Surveillance Committee (IRB) in accordance with institutional and federal regulations (45 CFR 46).

Clinical protocols are due in the Office of Protocol Research by 5:00 p.m. on the second or last Monday of the month. After receiving approval from the Clinical Research Committee, the Protocol will be acted upon by IRB.
Documentation of drug or device source approval is the responsibility of the study chairman. Source approval AND Surveillance Committee approval are required before a protocol can be activated.

1 Is this a Diagnostic or Therapeutic Study?	☐ Yes	☒ No
2 **Biosafety check:**		
Does this study include any products manufactured or produced at M. D. Anderson?	☐ Yes	☒ No
Does this protocol use recombinant DNA?	☐ Yes	☒ No
3 **Does this research involve the Use of Educational Tests, Survey Procedures, Interview Procedures or Observation of Public Behavior?**	☐ Yes	☒ No
Are responses recorded in such a manner that the subject can be identified, directly or through identifiers linked to the subject?	☐ Yes	☒ No
Could the subject's responses, if they became know outside the research, reasonably place the subject at risk of criminal or civil liability or be damaging to the subject's financial standing, employability, or reputation?	☐ Yes	☒ No
Does the research deal with sensitive aspects of the subject's own behavior, such as illegal conduct, drug use, sexual behavior, or use of alcohol?	☐ Yes	☒ No
4 Research Involving Human Specimens, Cell Lines or Data	☐ N/A	
a. **Human Specimens**	☒ Yes	☐ No
Will human specimens be obtained specifically for this research?	☐ Yes	☒ No
Will human specimens be obtained from incidental (residual) samples?	☒ Yes	☐ No
Are the data relating to the specimens kept by the investigator in such a way that the patient can be identified?	☐ Yes	☒ No
Does the investigator guarantee to preserve confidentiality?	☒ Yes	☐ No
Total number of patients 80 samples 160 required		
b. **Human Cell Lines** _____ **Data** _____	☐ Yes	☒ No
Are cell lines or data obtained from a public repository or source?	☐ Yes	☒ No
Can the cell lines or data be linked directly to the subject?	☐ Yes	☒ No
5 **Could the research lead to a patentable procedure or product?** (If yes, a copy of the proposal should be submitted to the Office of Technology Department, Box 510.)	☐ Yes	☒ No

The information provided regarding this research activity is true and correct to the best of my knowledge.

Title of Proposal: USE OF PARAFFFIN-EMBEDDED ARCHIVAL SAMPLES FROM PATIENTS WITH LEUKEMIA AND MYELODYSPLASTIC SYNDROMES TO STUDY DNA-METHYLATION.

Principal Investigator: _____ Box 61 Extension 5-3428
Guillermo Garcia Manero, M.D. 7/12/00

Collaborators: Jean-Pierre Issa, M.D. 7/12/00

Department Head Approval: _____
Hagop M. Kantarjian, M.D. 7/12/00

Surveillance Committee (IRB) _____ 7-20-00 Exemption 2 ____ Exemption 4 ____ Other lab
AMAN U. BUZDAR, M.D

need revised blue card

NON-EXEMPT HUMAN SUBJECTS RESEARCH
(NON-CLINICAL STUDIES)

Attach an abstract, optimally 1-3 pages, that provides an overview of the research you will be conducting whether or not it appears to be exempt from Surveillance Committee review.

Research that does not qualify for exemption must be approved by the Surveillance Committee. Protocol numbers will be assigned to the research proposals involving the use of human tissue, data, etc., and will be subject to the same annual review requirements as clinical studies. The protocol numbers and the dates of Surveillance Committee approval are essential to the award of research grants/contracts.

In order to receive Surveillance Committee review and a protocol number, the following points must be clearly addressed in your abstract so that the level of risk may be determined.

1 Provide a brief description of the research, including the objectives and a general description of the methodology employed.

2 Describe involvement of human subjects in the proposed research, including the characteristics of the subject population, their anticipated number of subjects, age, ranges, sex, ethnic background, and health status. Identify the criteria for inclusion or exclusion. Explain the rationale for the involvement of special classes of subjects, if any, such as fetuses, pregnant women, children, human in vitro fertilization, or others who are likely to be vulnerable.

3 Identify the sources of research material obtained from individually identifiable living human subjects in the form of specimens, records, or data. State whether the material or data will be obtained specifically for research purposes or whether use will be made of existing specimens, records, or data.

4 Describe plans for the recruitment of subjects and the consent procedures to be followed, including the circumstances under which consent will be sought and obtained, who will seek it, the nature of the information to be provided to perspective subjects, and the method of documenting consent.

5 Describe any potential risks - physical, psychological, social, legal, or other - and assess their likelihood and seriousness.

6 Describe the procedures for protecting against or minimizing any potential risks, including risks to confidentiality, and assess their likely effectiveness. As appropriate, discuss provisions for ensuring necessary medical or professional intervention in the event of adverse effects to the subjects. Also, where appropriate, describe the provisions for monitoring the date collected to ensure the safety of subjects.

7 Discuss why the risks to subjects are reasonable in relation to the anticipated benefits to subjects and in relation the importance of the knowledge that may reasonably be expected to result.

THE UNIVERSITY OF TEXAS
M.D. ANDERSON CANCER CENTER

DIVISION OF MEDICINE

USE OF PARAFFFIN-EMBEDDED ARCHIVAL SAMPLES FROM PATIENTS WITH LEUKEMIA AND MYELODYSPLASTIC SYNDROMES TO STUDY DNA-METHYLATION.

1-OBJECTIVES
2-BACKGROUND
3-METHODS
4-REFERENCES

PRINCIPAL INVESTIGATOR:

Guillermo Garcia-Manero, M.D.

CO-PRINCIPAL INVESTIGATOR:

Jean-Pierre Issa, M.D.

The University of Texas
M. D. Anderson Cancer Center
OFFICE OF PROTOCOL RESEARCH
PROTOCOL APPROVED
SIGNED: Colleen A Brunell
DATE: 9-8-00

1-Objectives.

-To study the DNA-methylation characteristics of multiple genes from patients with leukemia and myelodysplastic syndromes at diagnosis and relapse.

-To obtain the DNA needed to perform these studies from sections obtained from paraffin-embedded archival material.

2-Background

We are actively investigating the methylator characteristics of patients with ALL and have found important correlates between particular methylator phenotypes and prognosis in patients with this disease. We have used the AML-PO1 bank to perform these studies. Unfortunately the number of samples in this bank is limited. Access to archival material, mainly from bone marrow biopsies, will allow us to complete our studies, by analyzing remission and relapse marrow and expand our studies to other hematological malignancies. Results from these studies may lead to a new molecular classification of these disorders, the generation of new techniques to detect minimal residual disease and the development of new treatments using hypomethylating agents.

CpG methylation is the addition of a methyl group to the fifth position of the cytosine ring in a cytosine guanine dinucleotide (CpG). CpG sites are found in close proximity to the 5′end of many genes, involving both promoter regions and 5′ untranslated regions. The field of DNA methylation has generated considerable interest recently thanks to several conceptual and technical advances. First, the discovery that epigenetic gene silencing through DNA methylation plays a role in the development of several disease entities, including cancer, has triggered clinical interest in this phenomenon. Second, the development of new technologies to analyze the methylation state of multiple genes in a relatively short time, and to detect DNA methylation abnormalities in serum, has raised the hope of rapidly finding clinical applications for this research. Third, the cloning of several DNA methyltransferases, enzymes with the capacity of methylating DNA, and the discovery of mutations in one of these in a rare hereditary syndrome has increased interest in the role of the methylation machinery in disease. Finally, the possibility of reversing DNA methylation changes pharmacologically has opened new areas of research in the treatment and prevention of cancer.

Epigenetic silencing refers to stable, non-genetic inactivation of gene expression that is clonally inherited and thought to be irreversible under most normal situations (1). A role for DNA methylation in silencing was revealed by the observed inverse relationship between the transcriptional activity of a particular promoter and its methylation status (2). CpG island methylation related silencing is important for imprinting

(3)Besides this physiological role, aberrant CpG island methylation has been implicated in certain disease processes, such as the Fragile X syndrome (5), aging (6) and in cancer (7,8). DNA methylation has been implicated in oncogenesis by silencing the expression of genes crucial for cell function, such as tumor suppressor genes. This phenomenon represents a molecular alternative gene mutation, and/or deletion for the inactivation of critical genes. DNA methylation appears to induce silencing primarily by attracting a protein complex that includes methylated DNA binding proteins and histone deacetylases, ultimately resulting in the formation of a closed chromatin structure (9).

From the above information, it is clear that the analysis of the methylation state of specific genes in diverse disease entities can potentially provide useful clinical information, similar to the analysis of gene mutations or other abnormalities in these same disorders. For example, methylation of specific genes may provide prognostic information (10), and could potentially be used to screen for the presence of cancer. Therefore, the development of rapid and sensitive techniques to study DNA methylation is important for such translational research. Over the past few years, the study of DNA methylation has been greatly facilitated by the realization that bisulfite treatment of DNA results in the conversion of unmethylated cytosines to uracil, leaving most methylated cytosines intact (11). Several bisulfite-PCR based techniques have now been developed, that allow quantitative (12) or very sensitive (13) detection of gene specific methylation. These developments are providing a more complete picture of DNA methylation changes in cancer and their potential clinical implications.

The methylation analysis of multiple genes in individual patient samples has revealed considerable variation in the degree of methylation, suggesting that the process is not random, but likely results from specific defects in methylation control. Thus, an extensive analysis of colon cancer has revealed a subset of cases that evolves with a high degree of methylation and silencing – a phenotype termed CpG Island Methylator Phenotype (CIMP) (14). This concept has now been verified in other tumor types, including gastric (15) and hepatocellular cancer, as well as hematopoietic malignancies. The importance of this phenomenon is that the clinical and biological characteristics of the different methylation groups appear to be substantially different (16), and this may have clinical implications such as different prognosis, and different responses to specific therapeutic interventions.

3. Methods

3a-Samples and Patient confidentiality.

Samples will consist of archival material (paraffin embedded bone marrow biopsy) patients with known diagnoses of leukemia or myelodysplastic syndrome identified through the Department of Leukemia Patient Database. Initially, a list of patient identifiers by their history number will be submitted to the pathology department to obtain sections of paraffin-embedded tissues. These sections will be assigned a

laboratory number with no patient identities. The corresponding history number will only be known to the principal investigators, **thus preserving patient confidentiality.**

3b-DNA extraction. (17)

-Samples will be obtained from bone marrow biopsies.
-No tissue microdissection will be performed.
-10 μm sections will be cut from paraffin blocks and placed in a microfuge tube.
-Sections will then be suspended in digestion buffer containing proteinase K and incubated at 37C for 24 hours.
-After incubation, an equal volume of chloroform-isoamyl alcohol (24:1) will be added.
-Mix well and separate phases by centrifugation, the upper phase will be collected.
-Then 2 volumes of cold absolute ethanol and 1/10 vol. of 3 M sodium acetate will be added. DNA will be precipitated at –20C overnight.
-DNA is pelleted by centrifugation, dried and re-suspended in water.

3c-Bisulfite treatment of DNA.

-Our techniques to study DNA-methylation are described in the web site: **www.mdanderson.org\leukemia\methylation.** In summary, DNA is treated with bisulfite, this results in the conversion of unmethylated cytosines to uracil, leaving most methylated cytosines intact. Using specific PCR primers followed by DNA digestion by restriction enzymes, we can perform quantitative and very sensitive detection of gene specific methylation. The overall protocol is as follows: 1) bisulfite treatment of the DNA (this is performed over 16 hours); 3) PCR using specific primers designed based on the sequence of the gene specific CpG island and restriction site map; 4) Gel separation using polyacrilamide gels and 5) Image analysis using digital densitometric analysis. We can quantify the amount of methylation present in the CpG Island being amplified

4c-Analysis.

We plan to analyze the methylation status of multiple genes (MDR1, ER, THB1, THB2) in ALL, AML and MDS. The methylation status will

1) Be correlated with response, relapse rates and overall survival.
2) Be studied at diagnoses and relapse to determine whether methylation is a major mechanism of disease resistance.
3) Be studied at remission to determine whether minimal residual disease quantitation can predict early relapse in leukemia.

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